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13. ABSTRACT (Maximum 200 Words)
Our group previously showed that FOXO proteins are aberrantly localized to the cytoplasm in cells that have sustained loss of functional PTEN. Moreover, reconstitution of PTEN function to such cells leads to restoration of FOXO to the nucleus. To a first approximation, small molecules that recapitulate this activity of PTEN, i.e. lead to re-distribution of FKHR from the cytoplasm to the nucleus, should lead to inhibition of cell-cycle progression and suppression of tumorigenicity of PTEN null cells. These data led us to ask whether a novel cell-based small-molecule screen could be developed using FKHR localization as an end-point. Preliminary data showed that this was feasible and led to the discovery of novel small molecule inhibitors of the PI3K pathway. Based on these results we proposed 3 specific aims:

1) To determine the mechanism of action of inhibitors that specifically re-localize FKHR to the nucleus
2) To determine the in vitro biological activity of small molecule inhibitors discovered in the FKHR screen.
3) To determine the in vivo anti-tumor efficacy of lead compounds in animal models.

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INTRODUCTION

The *PTEN/MMAC/TEP-1* tumor suppressor gene (hereafter referred to as *PTEN*) is a target of somatic mutation in prostate cancer as well as in endometrial cancer, glioblastoma and melanoma (reviewed in (Sansal and Sellers, 2004)). Biallelic loss of *PTEN* has been demonstrated in both primary and metastatic prostate tumors (reviewed in (Sansal and Sellers, 2004)). In metastatic disease, *PTEN* loss approaches 50%-60% (Suzuki et al., 1998). Together, these data suggest that loss of PTEN is an important step for those prostate tumors associated with a lethal outcome. **Thus, understanding the mechanisms by which PTEN acts as a tumor suppressor, and developing novel therapeutics that target the PTEN pathway are essential areas of investigation.**

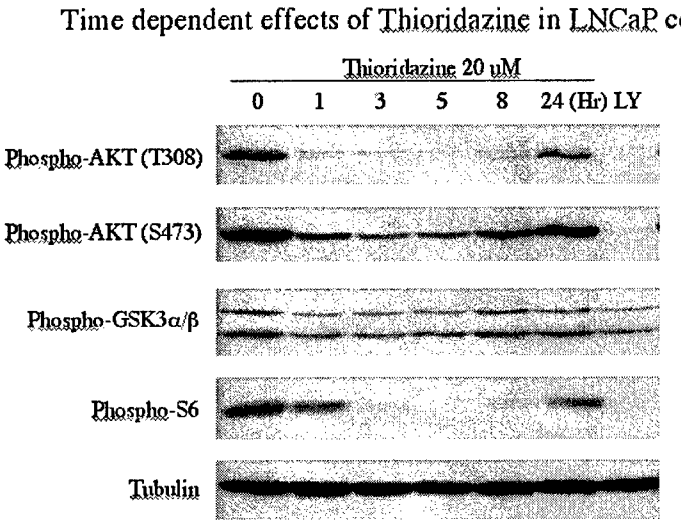

Figure 1: Time course of PI3K pathway inhibition after treatment with thioridazine (see text).

Based on the data that FKHR localization is aberrantly localized loss of functional PTEN and that FKHR localization is restored upon reconstitution of PTEN to PTEN null cells, to a first approximation, small molecules that recapitulate this activity of PTEN, i.e. lead to re-distribution of FKHR from the cytoplasm to the nucleus, should lead to inhibition of cell-cycle progression and suppression of tumorigenicity of PTEN null cells. These data led us to ask whether a novel cell-based small-molecule screen could be developed using FKHR localization as an end-point. Preliminary data showed that this was feasible and led to the discovery of novel small molecule inhibitors of the PI3K pathway (Kau et al., 2003). Based on these results we proposed 3 specific aims:

1) To determine the mechanism of action of inhibitors that specifically re-localize FKHR to the nucleus

2) To determine the in vitro biological activity of small molecule inhibitors discovered in the FKHR screen.

3) To determine the in vivo anti-tumor efficacy of lead compounds in animal models.

Body

Thioridazine decreases phospho-Ser473 and phospho-Thr308 Akt levels.

In order to clarify the phosphoinositide-3 kinase inhibitory activity of phenothiazines, the phosphorylation state of Akt and its downstream proteins were examined after treatment with thioridazine or other phenothiazine derivatives in both PTEN wild-type and PTEN mutant cell lines. To this end, extracts were prepared from PTEN null LNCaP cells grown in complete media at different time points after treatment with 20 μM thioridazine (Fig. 1). Treatment with thioridazine decreased phospho-Thr308-Akt levels from 1 hr to 8 hrs after treatment with peak activity seen at the 5-hour time point thioridazine. Phosphorylation of Ser473-Akt levels were abolished with a similar time course. To determine whether the loss of Akt phosphorylation was associated with loss of phosphorylation of downstream pathway effectors, protein extracts were probed with

antisera recognizing phosphorylated GSK3α/βand phosphorylated S6 ribosomal protein. Phosphorylation of GSK3α/β and S6 ribosomal protein were diminished with a similar time course. Next, LNCaP cells were treated with various concentration of thioridazine for 5 hrs and the protein extracts again were immunoblotted with the relevant phosphospecific antisera (Fig. 2). Treatment with thioridazine decreased the levels of phospho-Thr308-Akt, phospho- Ser473-Akt, phosphorylated GSK3 and phosphorylated S6 ribosomal in a dose dependent manner. To determine the cell based concentration of thioridizaine leading to 50% diminishment of Akt activity (Cellular IC50) the phosphorylation of Akt was quantified by determining the optical density of bands of phospho-Ser473- and phospho-Thr308-Akt on immunoblot (data not shown) analyzed by NIH-Image software and normalized with □nti-GSK3□. The thioridazine concentration producing 50% inhibition (IC50) of phospho-Ser473- and phospho-Thr308-Akt levels were 9.6 and 11.7 μM (data not shown) respectively. As we previously reported these data suggest that thioridazine can act at or upstream of Akt to inhibit its activity and inhibit pathway activity downstream of Akt.

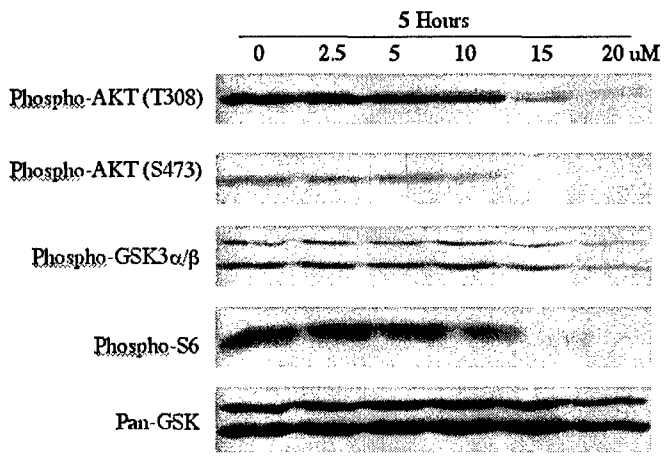

Figure 2: Dose-dependent inhibition of PI3K pathway signaling by phenothiazines (see text).

A prediction of these data is that PTEN null cells might be preferentially sensitized to cellular growth inhibitory effects of phenothiazine. To try and determine whether this was the case, we next examined the effect of phenothiazines in two pairs of cell lines, LNCaP and DU145 prostate cancer cell lines and 786-O and ACHN renal carcinoma cell lines (Fig. 2). LNCaP and 786-O cells fail to express any full length of PTEN protein, but DU145 and ACHN cells retain wild-type PTEN alleles and express an intact PTEN protein (Ramaswamy et al., 1999). In PTEN null cells, as previously noted by numerous groups including our own, Akt activity and phosphorylation of substrates such as FOXOs (FOXO3A) and GSK3 are substantially lower when compared to PTEN null cells. Treatment with 20 μM thioridazine for 5 hours decreased the phosphorylation of Akt, FKHRL1, and GSK3in both PTEN-null cells (LNCaP and 786-O). The phosphorylation state of p70 S6 kinase (p70 S6K) and S6 ribosomal protein were also inhibited and were notable in that both their phosphorylation and inhibition by thioridizines were not directly correlated with PTEN status. Specifically, under plating conditions where cells were plated at low-density PTEN wild-type DU145 and ACHN cells showed hyperphosphorylation of S6 ribosomal protein and p70S6K, which was rapidly blocked by treatment with Thioridazine (data not shown). Thus, thioridazine treatment led to decreased phosphorylation of p70 S6K and S6 ribosomal protein in all PTEN-null and PTEN wild-type cell lines tested, LNCaP, 786-O, DU145 and ACHN cells. The lack of robust Akt activation in the PTEN wild-type cells, and the inhibitory effect of thioridazine seen in the absence of Akt activity, suggests that phenothiazines may block pathway activation both upstream and downstream of Akt. Together with previous data linking the activity of phenothiazimes to calmodulin inhibition, these data raise the possibility that phenothiazines interdict a calmodulin dependent regulatory mechanism required both upstream and downstream of Akt in the PI3K pathway. Given the emerging data for a positive feedback loop induced by mTOR inhibition, a bispecific

inhibitor might be attractive. IC50 for growth showed that PTEN cells remain sensitized to the growth inhibitory effects of phenothiazines compared to normal cells.

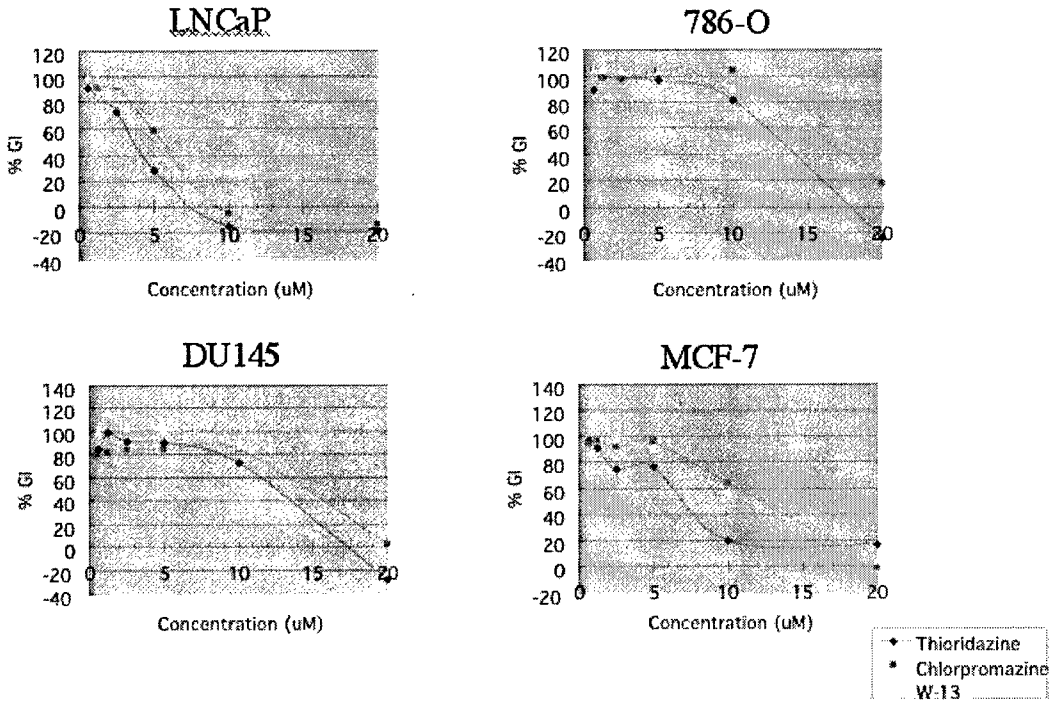

Figure 3: Cellular growth inhibitory concentrations of phenothiazimes and a calmodulin inhibitor in PTEN null and PTEN wild-type cells (see text).

The cells relatively sensitive to rapamycin are also sensitive to thioridazine.

PTEN null cells are known to be preferentially sensitized to mTOR inhibition (Aoki et al., 2001; Neshat et al., 2001; Podsypanina et al., 2001). To determine whether PTEN null cells similarly might be sensitized to growth inhibitory effects of phenothiazines, we tested the growth inhibitory effects of several phenothiazines, thioridazine, trifluoperazine, chlorpromazine, fluphenazine, and prochlorperazine, in the PTEN null PC-3 and U87MG cells. Among the tested phenothiazines, thioridazine showed most potent growth inhibitory effect in both cell lines (data not shown). To examine the inhibitory effect of thioridazine on the growth of PTEN-wild type vs. PTEN-null cells, we treated 6 cell lines that have been defined the PTEN status with varying dose of thioridazine. The concentration of thioridazine required for 50% growth inhibition (%GI50) for PTEN-null cell lines were lesser than for PTEN-wt cell lines except 786-O cells (data not shown). We tested the growth inhibitory effects of rapamycin in these cell lines and compared the growth inhibitory effects of thioridazine and rapamycin. Rapamycin is known as a potent mTOR inhibitor, a downstream protein kinase in the Akt pathway. Rapamycin showed strong growth inhibitory effect in PTEN-null cells but 786-O cells showed resistance to rapamycin (data not shown). Rapamycin sensitive cell lines, PC-3, LNCaP and U87MG cells, were relatively more sensitive to thioridazine than rapamycin resistant cell lines. 786-O which showed rapamycin resistance in

spite of PTEN-null status was also relatively resistant to thioridazine. This line in the NCI60 is reported as both sensitive, in one set of experiments, and insensitive in another. These similar sensitivity patterns of various cell lines which have different PTEN state to rapamycin and thioridazine would suggest that the growth inhibitory effect of thioridazine is likely linked to its PI3K pathway inhibitory activity. If thioridizaines have added therapeutic benefits resulting from the upstream inhibition of Akt, then the ability to block Akt phosphoryation might be expected to enhance the anti-cancer activity of rapamycin. On the other hand if most of the growth inhibitor effects result from inhibition of mTOR signaling we would expect non-synergistic or even non-additive activity. We tested the combination effect of thioridazine and rapamycin in LNCaP and DU145 cells (data not shown). We treated cells with the mixture of serially diluted concentration of rapamycin and fixed concentrations of thioridazine. The combination did not induce synergistic growth inhibition or result in any remarkable differences in sensitivity to rapamycin in LNCaP and DU145 cells.

In vivo testing of phenothiazines

As we go forward we are now trying to determine whether we can demonstrate anti-tumor activity of these compounds in animals. Specifically, we have previously generated and published our work developing a model of Akt activation in the murine prostate (Majumder et al., 2004; Majumder et al., 2003). We are now trying to determine whether we can inhibit Akt activation and reverse the PIN lesions seen in this model.

KEY RESEARCH ACCOMPLISHMENTS:

Bulleted list of key research accomplishments emanating from this research.
-have demonstrated preferential growth inhibition of PTEN null cells for the phenothiazime class of drugs.
-have shown that phenothiazines can act both upstream and downstream of Akt in the PI3K pathway.

REPORTABLE OUTCOMES:

Provide a list of reportable outcomes that have resulted from this research to include:

1. Kau, T. R., Schroeder, F., Ramaswamy, S., Wojciechowski, C. L., Zhao, J. J., Roberts, T. M., Clardy, J., Sellers, W. R., and Silver, P. A. (2003). A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell *4*, 463-476.

2. Sansal, I., and Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*, 2954-2963.

CONCLUSIONS:

We have demonstrated that phenothiazines have anti-tumor growth activity that is apparently dependent upon their ability to interrupt PI3K signaling. This activity appears to act both upstream of Akt and downstream of Akt making this a relatively unique therapeutic. It is likely that this dual activity reflects a requirement for

calmodulin activity at both points in the pathway.

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Sansal, I., and Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*, 2954-2963.

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APPENDICES:

1. Kau, T. R., Schroeder, F., Ramaswamy, S., Wojciechowski, C. L., Zhao, J. J., Roberts, T. M., Clardy, J.,

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A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells

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Summary

The PI3K/PTEN/Akt signal transduction pathway plays a key role in many tumors. Downstream targets of this pathway include the Forkhead family of transcription factors (FOXO1a, FOXO3a, FOXO4). In PTEN null cells, FOXO1a is inactivated by PI3K-dependent phosphorylation and mislocalization to the cytoplasm, yet still undergoes nucleocytoplasmic shuttling. Since forcible localization of FOXO1a to the nucleus can reverse tumorigenicity of PTEN null cells, a high-content, chemical genetic screen for inhibitors of FOXO1a nuclear export was performed. The compounds detected in the primary screen were retested in secondary assays, and structure-function relationships were identified. Novel general export inhibitors were found that react with CRM1 as well as a number of compounds that inhibit PI3K/Akt signaling, among which are included multiple antagonists of calmodulin signaling.

Introduction

The PTEN lipid phosphatase acts as a tumor suppressor and negative regulator of PI3K/Akt-driven cell growth and survival. It antagonizes PI3K signal transduction by dephosphorylating the PI3K phosphorylation products, PI3,4,P_2 (PIP2) and PI3, 4,5,P_3 (PIP3) (Maehama and Dixon, 1998). Mutations in PTEN have been implicated in Cowden Disease—a hereditary disease marked by a high predisposition for breast and thyroid cancers (Vazquez and Sellers, 2000). In addition, PTEN deficiency has been found in cancers such as glioblastoma multiforme, endometrial and prostate cancer, melanoma, and renal cell carcinoma (Kondo et al., 2001; Kong et al., 1997; Wang et al., 1997). Inhibition of the PI3K/Akt signaling pathway in PTEN null cells can control aberrant cell growth.

Mammalian members of the FOXO or Forkhead family of transcription factors include FOXO1a, FOXO3a, and FOXO4 (also known as FKHR, FKHRL1, and AFX, repectively), each of which are phosphorylation targets of Akt (Brunet et al., 1999; del Peso et al., 1999; Kops et al., 1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999). These transcription factors are involved in negatively regulating cell cycle progression and cell survival (Medema et al., 2000; Nakamura et al., 2000). The phosphorylation state and subsequent subcellular localization help regulate the activity of these factors. In PTEN mutant cells, increased PIP3 levels result in constitutive activation of Akt, which phosphorylates FOXO transcription factors at multiple sites, preventing transcriptional activity and promoting nuclear export (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999; Rena et al., 1999). In addition to Akt, there is growing evidence that other kinases such as SGK, DYRK1A, CK1, and PAK1 are involved in FOXO phosphorylation and export (Brunet et al., 2001; Mazumdar and Kumar, 2003; Rena et al., 2002; Woods et al., 2001). Phosphorylation of the FOXO transcription factors facilitates binding with 14-3-3 proteins and export out of the nucleus (Brunet et al., 2002; Rena et al., 2001). Nuclear export of these factors is mediated by the export recep-

SIGNIFICANCE

The PTEN gene is mutated in a significant number of tumors, leading to the loss of PTEN lipid phosphatase activity and constitutive activation of PI3K/Akt signaling. It is currently unclear as to where in this pathway one might seek to intervene with a small molecule inhibitor and whether novel targets for therapy might exist. Surprisingly, an unbiased cell-based, small molecule screen based on FOXO1a localization led to the discovery of several inhibitors, including those that inhibit calmodulin. These data suggest that this approach can lead to the identification of novel lead compounds and targets for therapeutic development against tumors in which the PI3K/Akt pathway is aberrantly activated.

tor CRM1 (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002).

Many proteins that exit the nucleus contain short stretches of amino acids that act as nuclear export sequences (NESs) (Gerace, 1995). The NES-bearing protein is bound in the nucleus by the nuclear export receptor, CRM1, and escorted to the cytoplasm via a channel formed by the nuclear pore complex (NPC) (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Leptomycin B (LMB) inhibits protein nuclear export mediated by CRM1 (Kudo et al., 1998). Isolated from Streptomyces, LMB covalently modifies CRM1 at a specific cysteine residue by a Michael-type addition which inhibits binding of CRM1 with the NES-containing cargo (Kudo et al., 1999). Thus, the NES-containing cargo becomes trapped in the nucleus. FOXO transcription factors are among those proteins whose nuclear export is inhibited by LMB (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002).

When the three known Akt phosphorylation sites on FOXO1a are mutated to alanine, this AAA mutant can no longer undergo phosphorylation and accumulates in the nucleus, allowing reconstitution of FOXO1a activity to PTEN null cells (Nakamura et al., 2000; Ramaswamy et al., 1999). Such reconstitution arrests cells in G1, inhibits soft-agar growth, and inhibits xenograft growth in nude mice, thus recapitulating many aspects of PTEN-mediated tumor suppression (Nakamura et al., 2000; Ramaswamy et al., 1999). Small molecules that block FOXO1a export could target members of the general protein transport machinery and inhibit proteins other than CRM1. In addition, small molecules may act as novel kinase inhibitors, revealing new aspects of FOXO1a signal transduction, and serve as preliminary anticancer therapeutics.

The present study describes a cell-based, chemical genetic screen using FOXO1a subcellular localization as the readout. Two classes of compounds that inhibit FOXO1a nuclear export were identified: (1) compounds that target the general nuclear transport machinery and (2) compounds specific to the PI3K/Akt/FOXO1a signaling pathway. Several compounds in the first class possess electrophilic moieties that most likely alkylate Cys528 in CRM1 by a manner similar to the mode of action of LMB. Compounds in the second class have been characterized further and implicate calmodulin as a mediator of FOXO1a nucleocytoplasmic localization and regulation.

Results

A cell-based screen for FOXO1a nuclear localization in PTEN null cells

In order to identify novel nuclear transport inhibitors as well as small molecules that target the PI3K/Akt/FOXO1a signaling pathway, a visual cell-based assay was developed that used FOXO1a subcellular localization as the output. PTEN null, 786-O renal carcinoma cells were infected with an adenovirus expressing FLAG epitope tagged FOXO1a (Ad-FKHR). Cells were grown for 24 hr after infection to allow for adequate expression of FLAG-FKHR and then treated with compounds for 1 hr, followed by immunostaining and imaging. In these cells, as previously published, FOXO1a is constitutively localized to the cytoplasm (Figure 1A). In contrast, FOXO1a localizes to both the nucleus and cytoplasm in PTEN$^{+/+}$ growing U2OS cells (Figure 1A). Treatment of infected cells with the PI3K inhibitor, wortmannin, led to FOXO1a relocalization to the nucleus and, likewise, treatment with the nuclear export inhibitor, LMB, also resulted in FOXO1a nuclear sequestration (Figure 1B). As a negative control, DMSO did not affect FOXO1a subcellular localization (Figure 1B). These data indicate that FOXO1a shuttles between the nucleus and cytoplasm in a PI3K- and CRM1-dependent manner and that the intracellular shuttling of FOXO1a is an appropriate measure of both PI3K/Akt pathway activation and CRM1 or possibly other nuclear export factor activation (Figure 1C).

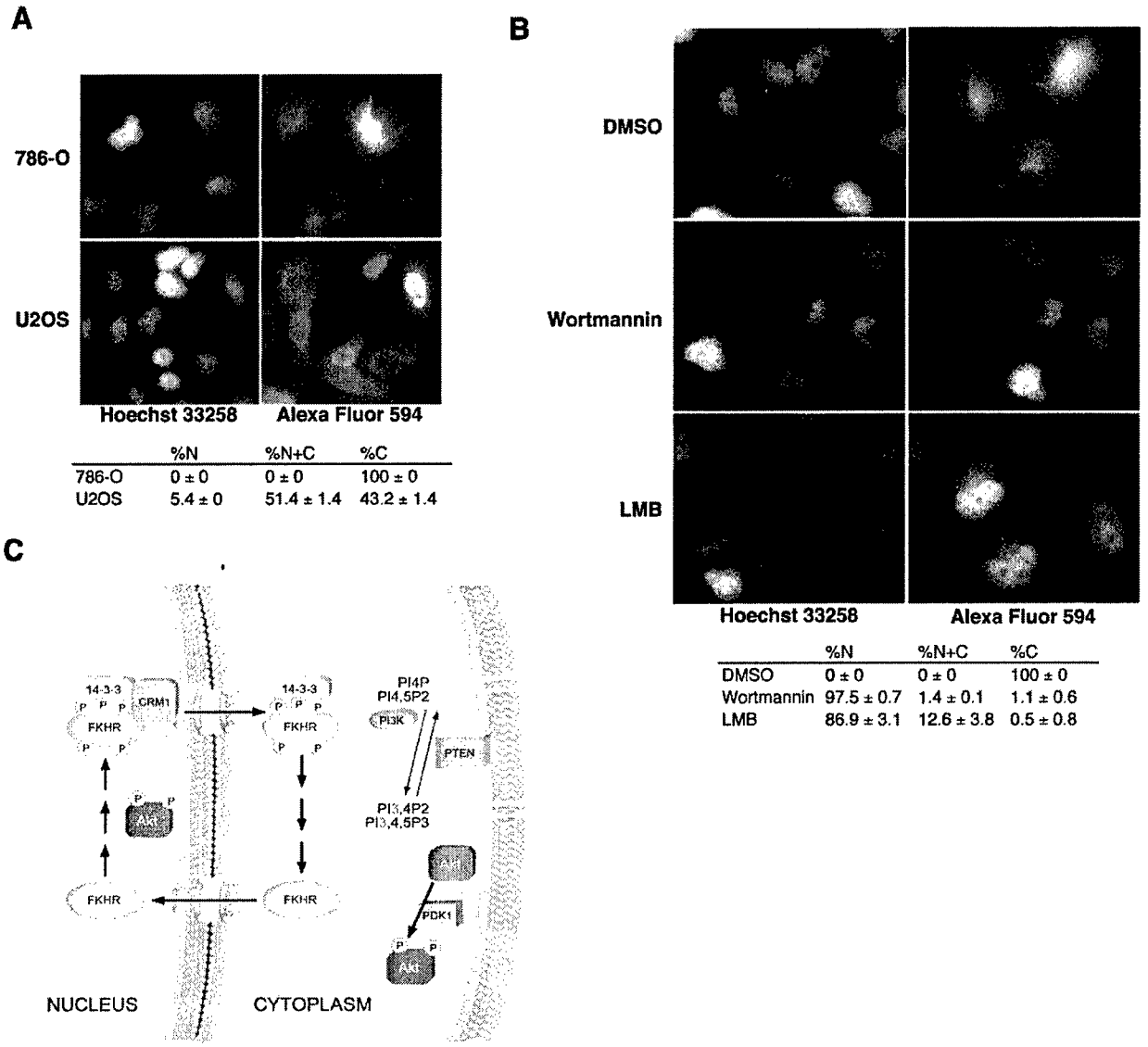
Using localization of FOXO1a as a visual assay, >18,000 compounds from the NCI Structural Diversity Set, ChemBridge DiverSetE, and a small collection of NCI marine extracts were tested for their ability to relocalize FOXO1a to the nucleus in PTEN null cells. Ad-FKHR-infected 786-O cells were grown on 384-well, clear-bottom plates before library compounds were transferred onto cells by a 384-pin array robot. Cells were fixed and stained after 1 hr treatment and FOXO1a localization determined by automated fluorescence microscopy. Eighty-nine compounds relocalized FOXO1a to the nucleus in this primary screen. Based on availability and potency, 42 total compounds were obtained for further characterization (seven from the NCI Structural Diversity Set, 34 from ChemBridge DiverSetE, and one from the NCI marine extracts).

Identification of general export inhibitors

In order to distinguish compounds that might target the general nuclear export machinery from those that target components of the PI3K/Akt/FOXO1a signaling pathway, each of the 42 small molecules identified in the primary screen was tested for the ability to block the export of HIV Rev, a protein known to undergo CRM1-dependent nucleocytoplasmic transport (Wolff et al., 1997). Here, compounds were serially diluted from 40 μM and added to U2OS cells stably expressing a RevGFP fusion protein containing the NES from PKI. As previously shown, this RevGFP fusion localizes to the cytoplasm at steady state (Figure 2A) (Henderson and Eleftheriou, 2000), and treatment of these cells with LMB leads to accumulation of RevGFP in the nucleolus (Figure 2A). Similarly, 19 of the initial 42 compounds blocked RevGFP export and are thus described hereforeward as "general export inhibitors" (Figure 2B).

The general export inhibitors target Cys528 of CRM1

LMB blocks NES-mediated nuclear export by covalently modifying CRM1 at Cys528 in humans and Cys529 in *S. pombe* by a Michael-type addition as illustrated in Figure 3A (Kudo et al., 1999, 1998). To determine whether any of these novel general export inhibitors similarly target the CRM1 Cys528 residue, U2OS-RevGFP cells were transfected with a dominant-negative CRM1 mutant expressing a Cys528Ser substitution. Cells expressing this mutant are viable and insensitive to LMB-mediated inhibition of nuclear export (Akakura et al., 2001) (Figure 3B). Furthermore, cells are still sensitive to LMB when overexpressing wild-type CRM1 (Akakura et al., 2001). Of the 19 general export inhibitors, 11 were inactive in CRM1-Cys528Ser transfected U2OS-RevGFP cells, suggesting that these compounds likely act by covalently modifying CRM1 at its reactive cysteine residue (Figure 3C). Approximately 50% of total cells exhibited cytoplasmic RevGFP—reflective of the transfection efficiency—in contrast to 100% nucleolar RevGFP in nontransfected cells. As a control, cells were also transfected with wild-type CRM1 and treated with the general export inhibitors. In these cells, the compounds blocked RevGFP export as they did in cells not overexpressing CRM1, as previously exhibited with LMB (data



	%N	%N+C	%C
786-O	0 ± 0	0 ± 0	100 ± 0
U2OS	5.4 ± 0	51.4 ± 1.4	43.2 ± 1.4

	%N	%N+C	%C
DMSO	0 ± 0	0 ± 0	100 ± 0
Wortmannin	97.5 ± 0.7	1.4 ± 0.1	1.1 ± 0.6
LMB	86.9 ± 3.1	12.6 ± 3.8	0.5 ± 0.8

Figure 1. FOXO1a subcellular localization and screening assay

A: PTEN$^{-/-}$ 786-O cells or PTEN$^{+/+}$ U2OS cells were infected with Ad-FKHR before FOXO1a cellular localization was visualized by immunofluorescence. In 786-O cells, FOXO1a is predominantly in the cytoplasm, whereas in U2OS cells, FOXO1a is both in the cytoplasm and nucleus. Nuclei were visualized by staining with Hoechst 33258. At least 200 cells were counted and the percent of predominantly nuclear (N), nuclear and cytoplasmic (N+C), and cytoplasmic (C) cells were determined with standard errors.

B: FOXO1a relocalizes to the nucleus in Ad-FKHR infected 786-O cells after treatment with wortmannin (39 nM) or LMB (4 nM), but not with DMSO.

C: Hyperphosphorylation of FOXO1a, as a result of PI3K/Akt signal transduction, promotes FOXO1a export into the cytoplasm. Inhibition of any one of the steps in the PI3K/Akt signaling pathway, as well as members of the nuclear export machinery, can lead to FOXO1a nuclear retention. PI3K phosphorylates PI4P or PI4,5P_2 and this reaction is reversed by the lipid phosphatase, PTEN. Upon PI3,4,5P_3 formation, Akt is recruited to the membrane and can undergo phosphorylation and activation by PDK1. FOXO1a is phosphorylated by an activated Akt, as well as other kinases, leading to binding with 14-3-3 and the promotion of nuclear export by CRM1.

not shown). The results for the remaining eight compounds were inconclusive in this assay. Thus, a second assay was developed in yeast to address the mode of action of these small molecules.

S. cerevisiae are insensitive to LMB because the yeast CRM1 contains a threonine at the homologous cysteine position (Neville and Rosbash, 1999). Cells can be made LMB sensitive by integration of a CRM1 mutant containing a Thr539Cys substitution in place of the wild-type yeast CRM1 (Neville and Rosbash, 1999). Based on these observations, wild-type yeast cells and cells containing the mutated CRM1 were transformed with a reporter plasmid expressing GFP containing both an NLS and an NES (Taura et al., 1998). In wild-type yeast cells, this protein shuttles between the nucleus and the cytoplasm, yet appears mostly in the yeast cytoplasm (Figure 3D). Wild-type yeast cells transformed with the NLS-NES-GFP reporter do not show accumulation of NLS-NES-GFP in the nucleus after treatment with LMB (Figure 3D). Conversely, in yeast cells expressing the CRM1 Thr539Cys mutation, shuttling of NLS-NES-GFP is

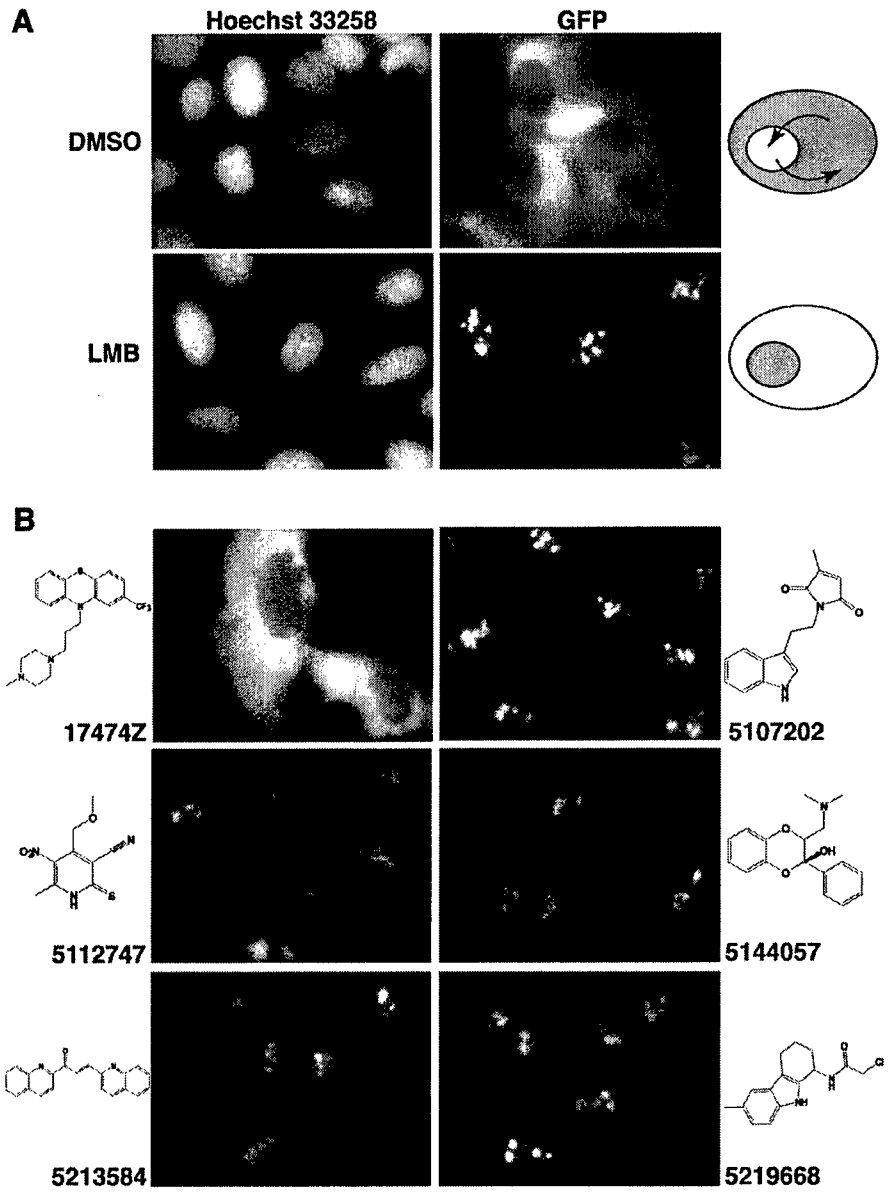


Figure 2. RevGFP export assay for general nuclear export inhibitors

A: U2OS cells stably expressing RevGFP were treated with DMSO or LMB. At steady state, RevGFP localizes to the cytoplasm in DMSO-treated cells while undergoing nucleocytoplasmic shuttling. However, in the presence of LMB (9.25 nM), RevGFP is trapped in the nucleoli due to a block in export in all cells. Nuclei were visualized by staining with Hoechst 33258.

B: U2OS-RevGFP cells were treated with all lead compounds resulting in 19 inhibitors of RevGFP export. Examples of five general export inhibitors at 20 μM trap RevGFP in the nucleoli of all cells (%N = 100) whereas the phenothiazine 17474Z at 20 μM does not (%N = 0).

blocked upon treatment with LMB. (Figure 3D). The 19 general export inhibitors, when retested in this assay, each blocked shuttling of NLS-NES-GFP in the humanized yeast. Thus, all appear to act through the CRM1 Cys528 residue (Figure 3E).

Compound structures reveal their mode of action

Comparing the structures of the 19 general export inhibitors to the structure of LMB revealed, in eight compounds, an α,β-unsaturated ketone or amide group that can likely undergo a Michael-type addition with the sulfhydryl group on Cys528 (Figure 4A; red). In addition, other compounds might undergo nucleophilic attack by the sulfhydryl group through a good halide leaving group (Figure 4A; blue), or across a triple bond (Figure 4A; green), or might undergo rearrangement for further reactivity (Figure 4A; pink). For three compounds, an obvious mechanism was not apparent though each was electrophilic (Figure 4A; black). Thus all of the general export inhibitors exhibit properties that likely explain the dependency of export inhibition on the CRM1 Cys528 residue.

Pathway-specific FOXO1a export inhibitors

Twenty-three compounds, though inhibitors of FOXO1a export, failed to alter export of RevGFP, and thus are termed pathway- or FOXO1a-specific. To determine whether such compounds were likely to act upstream or downstream of Akt, extracts were prepared from 786-O cells after treatment with each of the pathway-specific inhibitors or wortmannin, and phospho-Ser473-Akt was detected by immunoblotting. Two inhibitors (5219657 and B6-7-1) did not abolish phospho-Ser473-Akt levels similar to DMSO and thus likely have targets downstream of Akt or in a separate synergistic pathway (Figures 5A and 6). However, 21 compounds inhibited Ser473-Akt phosphorylation with varying

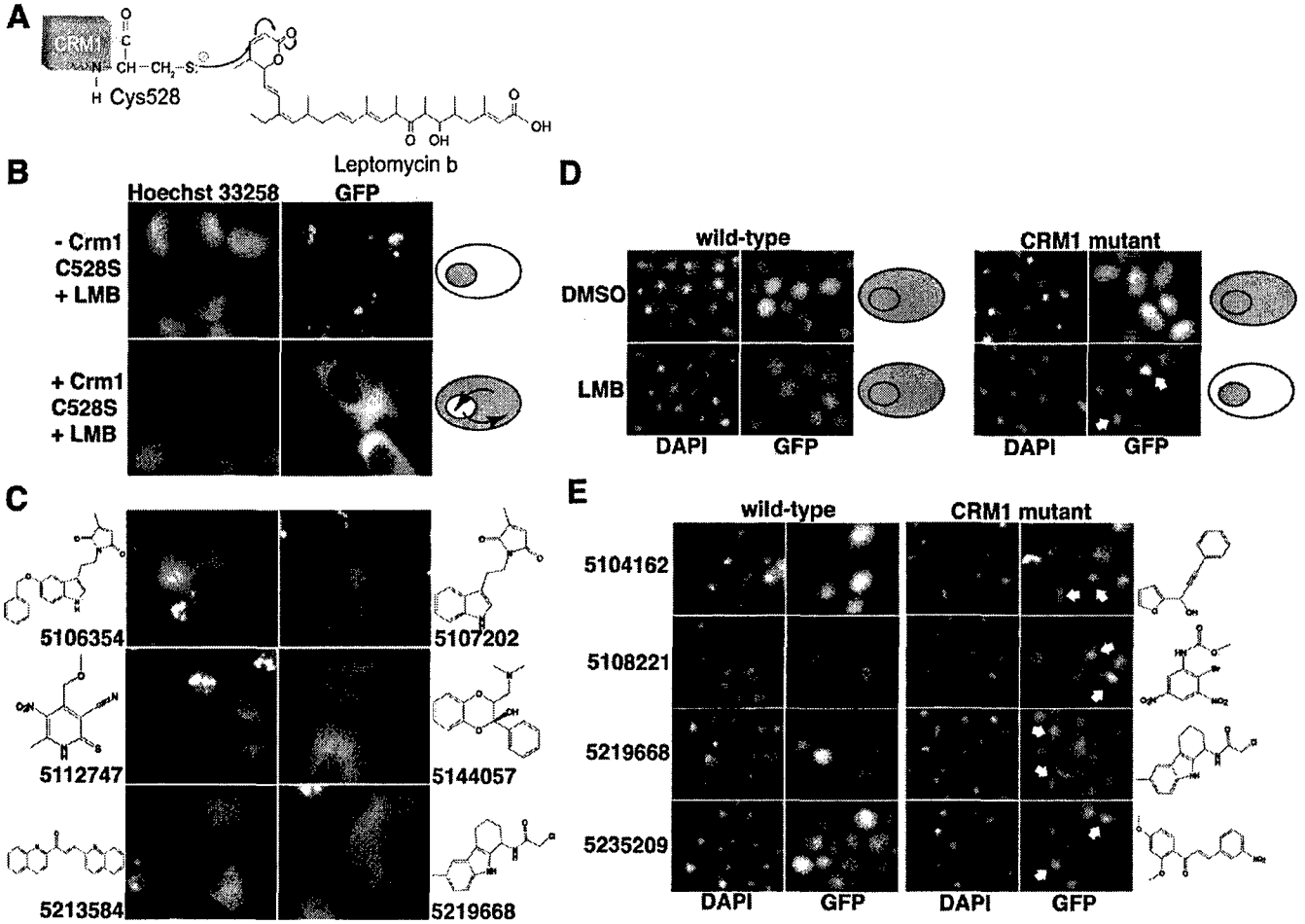


Figure 3. Assay for small molecules that target CRM1 in U2OS-RevGFP cells and yeast

A: LMB inhibits CRM1-mediated export by covalently modifying Cys528 by a Michael-type addition.

B: U2OS-RevGFP cells were either treated with 9.25 nM LMB or first transfected with the CRM1-Cys528Ser mutant before treatment with LMB. LMB blocks RevGFP export in nontransfected cells but does not block RevGFP export in cells expressing the CRM1-Cys528Ser mutant, resulting in about 50% cytoplasmic cells. Nuclei were visualized by Hoechst 33258 staining.

C: U2OS-RevGFP cells transfected with CRM1-Cys528Ser were treated with all 19 of the general export inhibitors. Here are examples of six compounds at 20 μM that do not block RevGFP export in cells expressing the CRM1 mutant, and thus target CRM1 at Cys528. About 50% of cells exhibited cytoplasmic RevGFP.

D: Wild-type yeast cells and humanized, mutant LMB-sensitive yeast cells were transformed with the NLS-NES-GFP reporter. NLS-NES-GFP is found throughout wild-type cells treated with either DMSO or 9.25 nM LMB. However, in CRM1 mutant, LMB-sensitive cells, 9.25 nM LMB treatment results in the nuclear accumulation of NLS-NES-GFP. DMSO treatment results in the reporter found throughout the cell. Nuclei were visualized by staining with DAPI.

E: All 19 of the general export inhibitors target CRM1 at the reactive cysteine residue when tested in wild-type and CRM1 mutant, LMB-sensitive yeast cells. Examples of various compounds (all at 20 μM) show NLS-NES-GFP localization throughout the cell in wild-type yeast and nuclear localization in CRM1 mutant cells. Arrows point to nuclear retention and nuclei were stained with DAPI for comparison.

efficacy. Phospho-Thr308 Akt was also probed and all compounds that blocked Ser473 phosphorylation similarly blocked Thr308 phosphorylation (Figures 5A and 6). As a control, treatment of cells with the general export inhibitors did not substantially alter Akt phosphorylation when tested at 20 μM, though three exhibited modest inhibition at 40 μM (Figure 6 and data not shown). Phospho-Ser255/Thr256 SGK levels were also probed revealing two compounds that significantly inhibited SGK phosphorylation (Figure 6). The two compounds that showed an effect, 5175309 and 5217339, might either exhibit less specificity or perhaps affect a parallel pathway leading to SGK and FOXO1a phosphorylation.

Akt nuclear localization was investigated in 786-O cells treated with pathway-specific inhibitors. If FOXO1a is phosphorylated by Akt in the nucleus, then it is possible that any compounds decreasing Akt phosphorylation levels and trapping FOXO1a in the nucleus might also result in the inhibition of FOXO1a phosphorylation by Akt mislocalization. Interestingly, treatment with the pathway-specific inhibitors did not affect Akt nuclear localization and overexpressed Akt as well as endogenous Akt was found throughout the cell (data not shown).

The pathway-specific compounds were next validated in a second assay in which FOXO1a localization is constitutively cytoplasmic based on expression of activated PI3K. Here, hu-

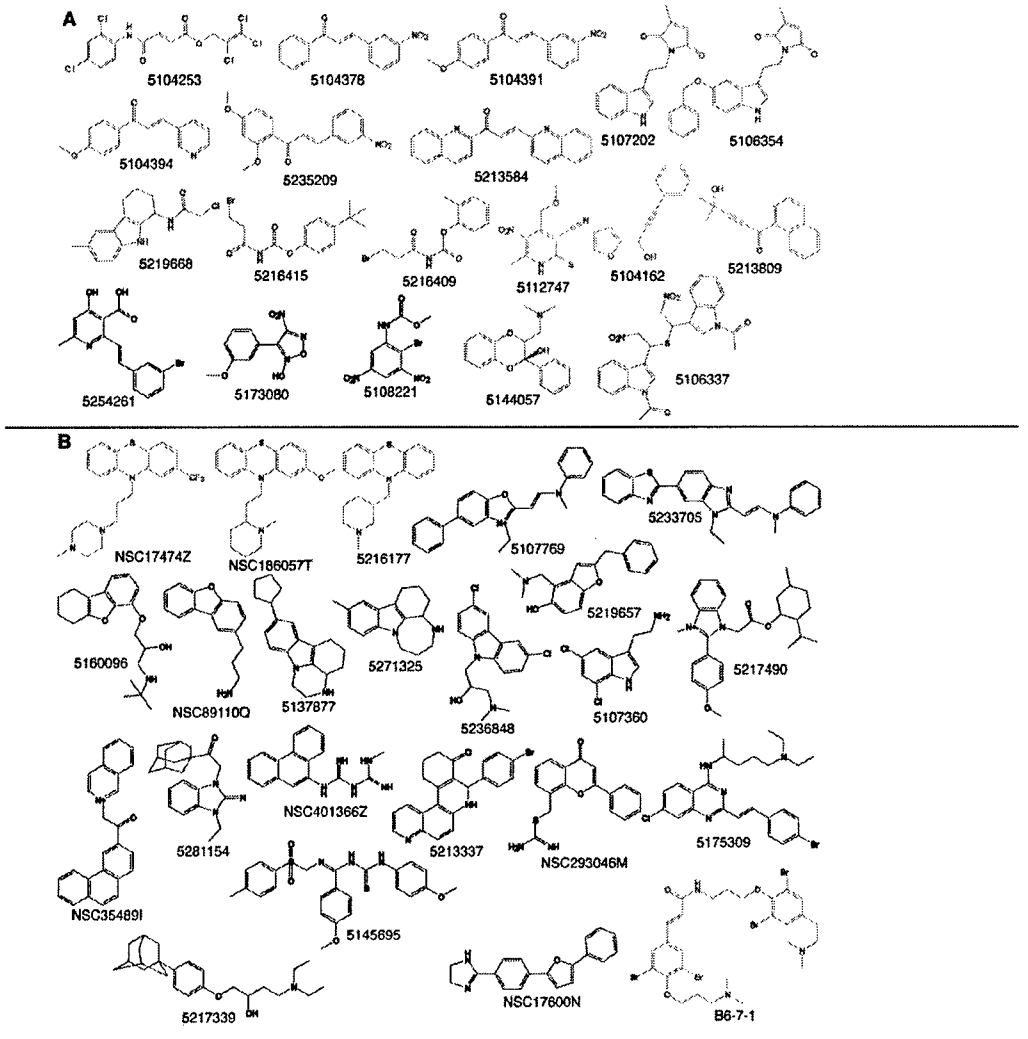


Figure 4. Structures of all lead compounds obtained for further characterization

A: General export inhibitors can covalently modify CRM1 like LMB either through an α,β-unsaturated ketone or amide as shown by compounds highlighted in red. Another method might be through the substitution of a good leaving group as shown by compounds highlighted in blue. Green highlighted compounds contain a triple bond, which may undergo nucleophilic addition. Compounds in pink might undergo rearrangement for further reactivity.
B: Structures of PI3K/Akt/FOXO1a specific inhibitors. Compounds in red belong to the structural family of phenothiazines. Several compounds contain planar heterocyles and may target the ATP binding sites of kinases. B6-7-1, in pink, was isolated from marine sponge extract.

man mammary epithelial cells (HMECs) containing either vector alone or stably expressing a membrane-targeted and, hence, constitutively active p110α subunit of PI3K (HMEC-Myr-p110α) (Zhao et al., 2003) were infected with Ad-FKHR. FOXO1a localization was then determined by immunofluorescence after treatment for 1 hr with each pathway-specific compound. In both cell types, FOXO1a is cytoplasmic. Thus the vector cells serve as a positive control for inhibition by a compound at any point along the PI3K pathway as well as a positive control for cell-specific drug metabolic properties such as cell permeability. Nine compounds that scored repeatedly in 786-O cells failed to score in the vector control cells, and thus their position in the pathway with respect to PI3K could not be formally established in this assay (Figures 5B and 6). Two compounds (89110Q, 293046M) blocked FOXO1a export in control cells, but not in HMEC-Myr-p110α cells, suggesting that these compounds have targets upstream of PI3K (Figures 5B and 6). Wortmannin and twelve pathway-specific inhibitors sequestered FOXO1a in the nucleus in both the vector and HMEC-Myr-p110α cells and thus likely act downstream of PI3K (Figures 5B and 6).

In order to determine whether structure-activity relationships existed with respect to these defined phenotypes, the structures of all pathway-specific inhibitors were examined. Several of these compounds contain planar heterocycles that might target the ATP binding sites of kinases. Examples include benzimidazoles (5217490, 5233705), an indole (5107360), a carbazole (5236848), hexahydrocarbazoles (5137877, 5271325), a quinazoline (5175309), a dihydrophenanthroline (5213337), a benzoxazole (5107769), benzofurans (5219657, 5160096), and a dibenzofuran (89110Q) (Figure 4B). B6-7-1, from a marine sponge extract, is an amino acid derived, novel bromotyrosine derivative (Figure 4B; pink).

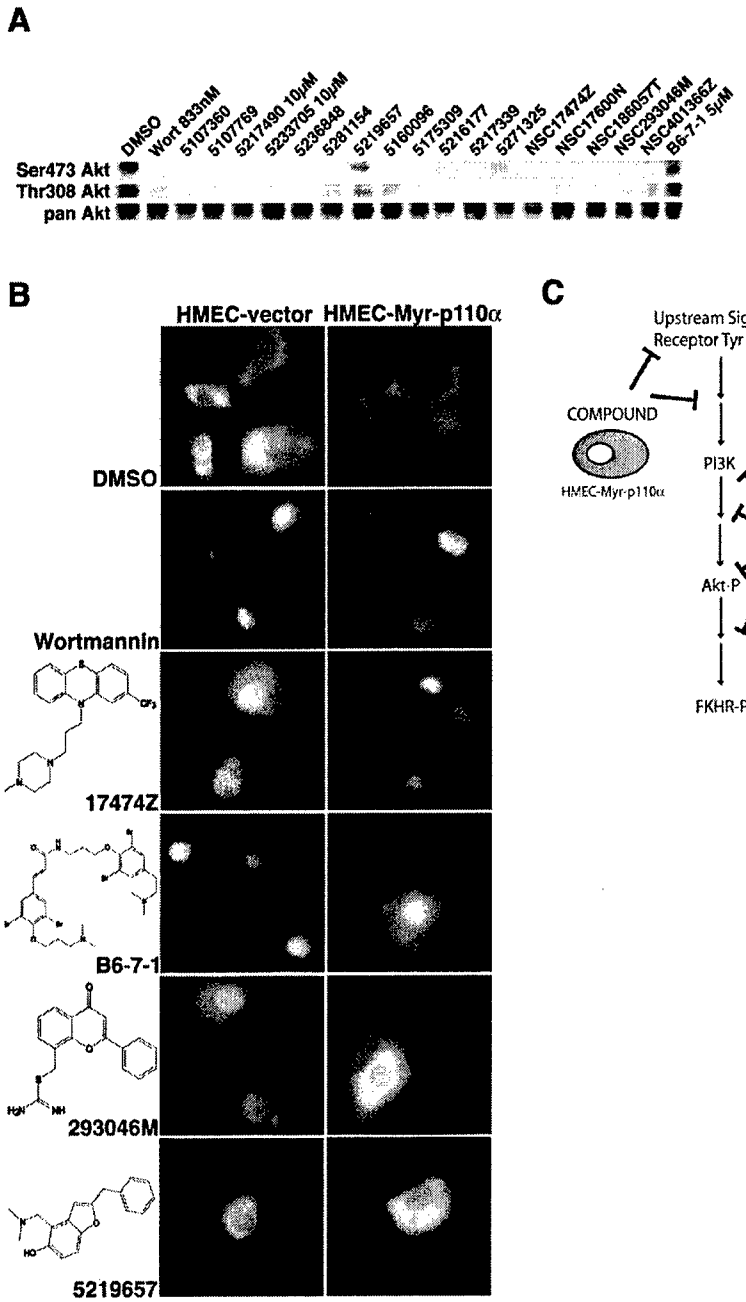


Figure 5. Most pathway-specific inhibitors decrease phospho-Ser473 and phospho-Thr308 Akt levels in 786-O cells, and block FKHR export in HMEC-Myr-p110α cells

A: Cell extracts were made from 786-O cells after treatment with pathway-specific compounds for 1 hr (all at 20 μM unless otherwise noted), and then immunoblotted with phospho-Akt and pan Akt antibodies.

B: FOXO1a localization in HMECs stably expressing Myr-p110α or vector control. HMECs were infected with Ad-FKHR and treated with pathway-specific compounds for 1 hr. Treatment with DMSO resulted in cytoplasmic FOXO1a in both HMEC-vector and HMEC-Myr-p110α cells. Treatment with 20 μM wortmannin, 40 μM trifluoperazine (17474Z), and 5 μM B6-7-1 results in nuclear FOXO1a in both cell types. Compound 293046M (80 μM) is an example of an inhibitor that causes FOXO1a nuclear localization in vector cells but not in Myr-p110α cells. Compound 5219657 (80 μM) is an example of an inhibitor that blocks FOXO1a export in neither cell types.

C: Compounds that trap FOXO1a in the nucleus in Myr-p110α cells target at or downstream of PI3K. Compounds that target upstream of PI3K block FOXO1a export in HMEC-vector cells but not in HMEC-Myr-p110α cells.

Phenothiazines—a class within the pathway-specific inhibitors

Of particular interest was the finding that three of the pathway-specific inhibitors belonged to the structural family of phenothiazines (Figure 4B; red) (17474Z, 186057T, 5216177), one of which was trifluoperazine (17474Z). Trifluoperazine, as well as many other phenothiazines, act as dopamine receptor antagonists and are clinically useful as antipsychotic and antiemetic medications. Like wortmannin, the three phenothiazines relocalized FOXO1a in 786-O cells to the nucleus, decreased phospho-Akt levels (Figure 5A), and in two instances blocked export in HMEC Myr-p110α cells (Figures 5B and 6).

To determine whether these activities were representative of the broader class of phenothiazines, additional phenothiazines including chlorpromazine, prochlorperazine, fluphenazine, and thioridazine were tested in the FOXO1a export assay. All of these compounds blocked FOXO1a export in 786-O cells (Figure 7A), raising the possibility that FOXO1a nuclear localization might be regulated by dopamine receptor signaling. Indeed, phospho-Akt levels increase when cells are treated with dopamine receptor agonists such as quinpirole and bromocriptine in neuronal cells (Brami-Cherrier et al., 2002; Kihara et al., 2002).

To investigate whether inhibiting the dopamine receptor can lead to FOXO1a nuclear retention, structurally unrelated dopamine receptor antagonists were tested in the FOXO1a export assay. None of the inhibitors—haloperidol, clozapine, L745870,

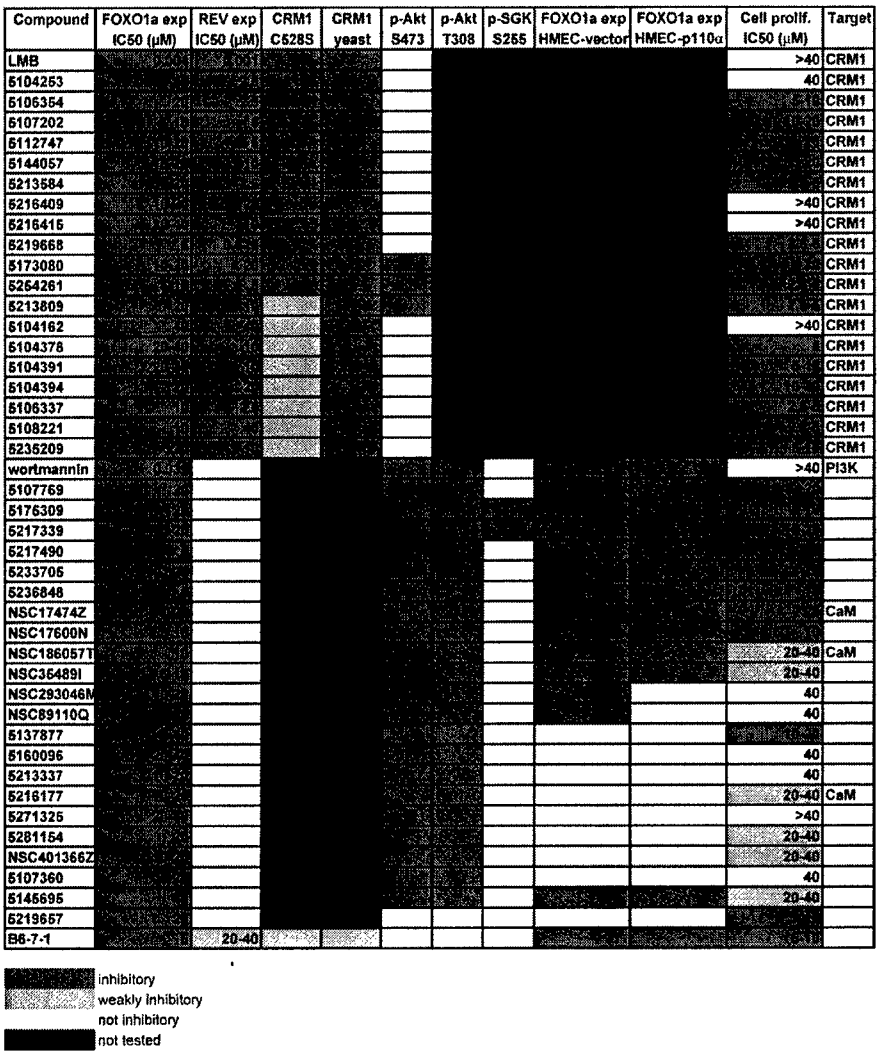


Figure 6. Collective assay results for all lead compounds tested

Compound numbers beginning with "5" are the ID numbers from ChemBridge and those beginning with "NSC" are the ID numbers from the NCI. Dark gray denotes the compound scored in the assay (e.g., blocks FOXO1a or RevGFP export; inhibits CRM1 in U2OS-RevGFP transfected cells or in the yeast NLS-NES-GFP export assay; decreases phospho-protein levels; blocks cell proliferation). Light gray denotes the compound has weak activity in the assay and white denotes the compound showed no activity in the assay. Black denotes the compound was not tested in the assay.

or L-stepholidine (L-SPD)—significantly blocked FOXO1a export at concentrations comparable to those previously used to block dopamine receptors in cells (Dong et al., 1997; Patel et al., 1997; Vanhauwe et al., 2000) (Figure 7B). These data strongly suggest that the dopamine receptor is not the relevant target of the phenothiazines with respect to their activity as inhibitors of FOXO1a export.

Structurally unrelated CaM inhibitors block FOXO1a export

Trifluoperazine (17474Z), in addition to its dopamine receptor antagonist activity, also exhibits inhibitory activity against calmodulin (Levin and Weiss, 1976, 1977), raising the possibility that it and the phenothiazine class as a whole might interrupt PI3K signaling through calmodulin inhibition. To ask whether this was the case, Ad-FKHR infected 786-O cells were treated for 1 hr with the calmodulin inhibitors W-13, calmidazolium, and ophiobolin A. Each of these inhibitors relocalized FOXO1a to the nucleus (Figure 7C). Moreover, when tested in serial titrations, FOXO1a export was blocked at concentrations comparable to those previously reported for CaM inhibition by these compounds in cells (Mottet et al., 2003; Wei et al., 1983; Yang et al., 2000). Treatment with the Ca^{2+} chelator, BAPTA-AM, also blocked FOXO1a nuclear export at 80 μM (Figure 7C). However, treatment with EGTA, an extracellular calcium chelator, did not result in FOXO1a nuclear localization (data not shown). These data are consistent with the notion that CaM activity regulated by intracellular Ca^{2+} regulates FOXO1a subcellular localization.

786-O cell proliferation in presence of compounds

IC50s were determined for all 42 lead compounds in cell proliferation studies. In these studies, 786-O cells were grown in 96-well plates and treated for ~24 hr at decreasing concentrations of inhibitor serially diluted in complete media from 40 μM to 1.25 μM. Cell viability was determined by correlation with amount of ATP released when cells were lysed. The results for these experiments are reported in Figure 6, which also summarizes the results of the assays for all general export and pathway-specific inhibitors. Most of the inhibitors block cell proliferation; however, ten compounds exhibited no effect at 40 μM, including wortmannin. It is possible these compounds, as with wortmannin, are unstable in aqueous solution for an extended period of

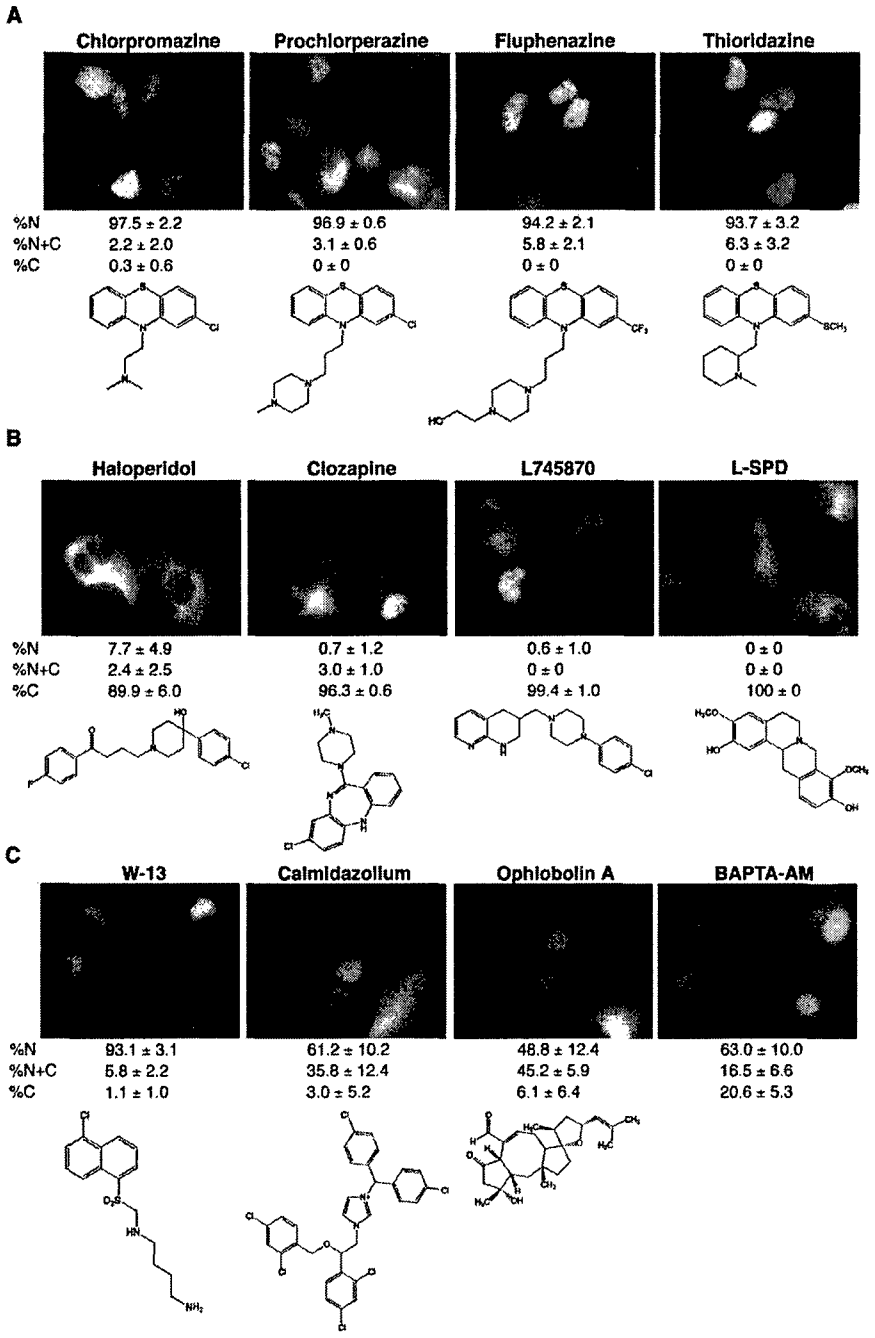


Figure 7. Trifluoperazine and other phenothiazines regulate FOXO1a subcellular localization through inhibition of CaM

A: 786-O cells infected with Ad-FKHR were treated with phenothiazine therapeutics. Chlorpromazine, prochlorperazine, fluphenazine, and thioridazine all relocalize FOXO1a to the nucleus at 20 μM.

B: Treatment with structurally unrelated dopamine receptor antagonists haloperidol (80 μM), clozapine (20 μM), L745870 (80 μM), and L-sperindone (80 μM) do not inhibit FOXO1a export significantly.

C: Treatment with structurally diverse CaM inhibitors W-13 (40 μM), calmidazolium (20 μM), and ophiobolin A (5 μM) block FOXO1a export. BAPTA-AM, an intracellular Ca^{2+} chelator, inhibits FOXO1a export at 80 μM.

time (Stein and Waterfield, 2000; Woscholski et al., 1994). Thus, compounds that localize FOXO1a to the nucleus after 1 hr may not inhibit proliferation in this 24 hr assay.

Discussion

A cell-based, visual, chemical genetic screen for small molecule inhibitors of nuclear export of the transcription factor FOXO1a was carried out in cancer-derived cells lacking functional PTEN protein. The utility of this "phenotypic" screen was validated by the discovery of small molecules specifically targeting key cellular proteins, and as such, 19 novel CRM1 export inhibitors were identified. Furthermore, a calmodulin-dependent regulatory mechanism for controlling FOXO1a localization was discovered. These data suggest that such screens, when conducted in defined systems, can be highly informative and likely compliment traditional in vitro target-based drug screens.

We have shown that 19 of the compounds that promote retention of FOXO1a in the nucleus are novel general protein export inhibitors. All 19 block the nuclear export of RevGFP

and FOXO1a by targeting CRM1, and not by inhibition of other factors in the nuclear transport machinery. Interestingly, all of these general inhibitors were isolated from the ChemBridge DiverSetE and make up about half of the total hits from that library. While most, if not all, of the general blockers are reactive, not all of the reactive compounds in the library relocalized FOXO1a in this screen, suggesting that the compounds identified in the screen exhibit some selectivity for CRM1. Of the 19 compounds in this class, 5219668 is the most potent CRM1 inhibitor (Figure 6). While not as potent as LMB, in some cases this compound can make a suitable substitute when inhibition of general export is required in experiments.

Surprisingly, targeting of CRM1 by the general export inhibitors invariably required the Cys528 residue and the chemical structures suggest that this dependency likely results from covalent interactions. On the other hand, the Cys528 residue is not required for CRM1 function and the three-dimensional structure of CRM1 has not been determined. Therefore, the specific mechanism by which covalent attack on this residue leads to inhibition is not known. The diversity of this new expanded set of CRM1 inhibitors may shed light on this question. Specifically, while it appears that Cys528 binding is required, it may not be sufficient. Thus, determining the structure-activity requirements of the reactive compounds scoring in this assay may define any additional requirements.

Although *S. cerevisiae* express a form of CRM1 that does not contain the reactive cysteine residue, *S. pombe*, human, and most other organisms do. The subselection of specific reactive compounds interacting with CRM1 begs the question of whether the export receptor CRM1 can act as a general sensor for certain types of reactive compounds in nature. For example, cellular and transcriptional responses to small molecule toxins present in the environment might be mediated through CRM1 binding and inactivation. Such inactivation would render CRM1 unable to export NES-containing proteins and certain transcription factors, including FOXO proteins. Trapped in the nucleus, these proteins might then enact transcriptional programs resulting in a cell cycle arrest or apoptosis, as examples. In this light, CRM1 might act as a protector of the cell.

The utility of LMB as a therapeutic has been explored with respect to the role of CRM1 in regulating p53 localization. For example, trapping p53 in the nucleus with LMB results in its reactivation and transduction of its biological responses including cellular apoptosis (Hietanen et al., 2000; Lain et al., 1999a), leading some to propose targeting nuclear transport as a means of controlling cancer growth (Kau and Silver, 2003; Lain et al., 1999b). In phase I clinical trials, however, targeting CRM1 with LMB was associated with profound toxicity (Newlands et al., 1996). Cells expressing mutant CRM1 are resistant to LMB; thus, it is critical to understand whether patient-related toxicity is target related or due to non-CRM1, off-target effects of LMB. If patient-related toxicity is linked to non-CRM1 effects of LMB, then the possible lower toxicity of some of the compounds identified in this screen could potentially guide the preclinical development of CRM1 inhibitors with improved therapeutic index.

One of the inherent strengths of our FOXO1a-based screen is the ability to identify both general export inhibitors and more specific effectors of the signaling pathways that promote FOXO1a movement and activity. Thus, 23 small molecule inhibitors of FOXO1a export that appear to act in the PI3K/Akt/FOXO1a signaling pathway were identified. Among these were a novel natural product from marine sponge and several compounds containing planar aromatic heterocycles similar to the scaffolds of known kinase inhibitors. These latter compounds may potentially inhibit the activity of kinases involved in Akt signaling or FOXO1a phosphorylation and export. Experiments designed to localize the inhibitory activity of these compounds within the pathway are ongoing.

Three of the pathway-specific compounds belong to the structural family of phenothiazines, including trifluoperazine, a known dopamine receptor antagonist and CaM inhibitor. While trifluoperazine and several other phenothiazine dopamine receptor antagonists relocalized FOXO1a to the nucleus, structurally unrelated antagonists such as haloperidol, clozapine, L745280, and L-SPD did not. Trifluoperazine and other phenothiazines such as fluphenazine and chlorpromazine also inhibit CaM in addition to dopamine receptors. Additional structurally diverse calmodulin inhibitors such as W-13, calmidazolium, and ophiobolin A robustly relocalized FOXO1a to the nucleus. Furthermore, treatment of 786-O cells with the intracellular Ca^{2+} chelator, BAPTA-AM, likewise relocalized FOXO1a to the nucleus, strongly suggesting that calmodulin is a regulator of FOXO1a cellular location. Interestingly, the natural marine sponge product, B6-7-1, is a bromotyrosine and resembles the bastadins. Bastadins have been shown to modulate ryanodine receptors, Ca^{2+} release channels in the sarcoplasmic reticulum (Mack et al., 1994). Though untested, it is possible that B6-7-1 might block Ca^{2+} channels and thereby reduce the intracellular Ca^{2+} concentration. Such an effect, as mimicked by our experiment with BAPTA-AM, might prevent Ca^{2+} from binding to CaM, inactivate CaM, and therefore keep FOXO1a nuclear.

Reducing intracellular Ca^{2+} levels or inhibiting CaM leads to inactivation of Akt in PC12 cells (Egea et al., 2001). Similarly, BDNF activation of Akt is blocked in cells expressing CaM with mutant Ca^{2+} binding domains (Cheng et al., 2003). The calmodulin inhibitor, W-13, while unable to alter PI3K or Akt kinase activity in vitro, decreases phospho-Akt levels in adipocytes in a PI3K-independent manner (Egea et al., 2001; Yang et al., 2000). Together, these suggest the presence of a CaM-dependent, PI3K-independent path to Akt activation. Finally, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) can phosphorylate Akt and SGK1 through a Ca^{2+}/CaM signaling pathway (Imai et al., 2003; Yano et al., 1998). Thus, one possibility is that FOXO1a cellular localization and activity might be regulated by a Ca^{2+}/CaM signaling cascade that results in the activation of CaM-KK and phosphorylation of both Akt and SGK1 by CaM-KK.

A schematic of the pathway depicting the site of action for the compounds that we have characterized thus far is shown in Figure 8. Several of the compounds might prove to be leads for potential anticancer drug development including trifluoperazine and 5233705. There is an ongoing vigorous debate on the virtues or perils of cell-based screening. This particular screen proved robust, facile, highly amenable to academic scale screens, and more importantly, elucidated previously undiscovered export and PI3K pathway inhibitors. Furthermore, we have determined the specific molecular mechanism for inhibition of representative compounds within both classes of inhibitors, leading to the discovery of novel CRM1 inhibitors and revealing the role of CaM in regulating PI3K signaling to FOXO1a. Experi-

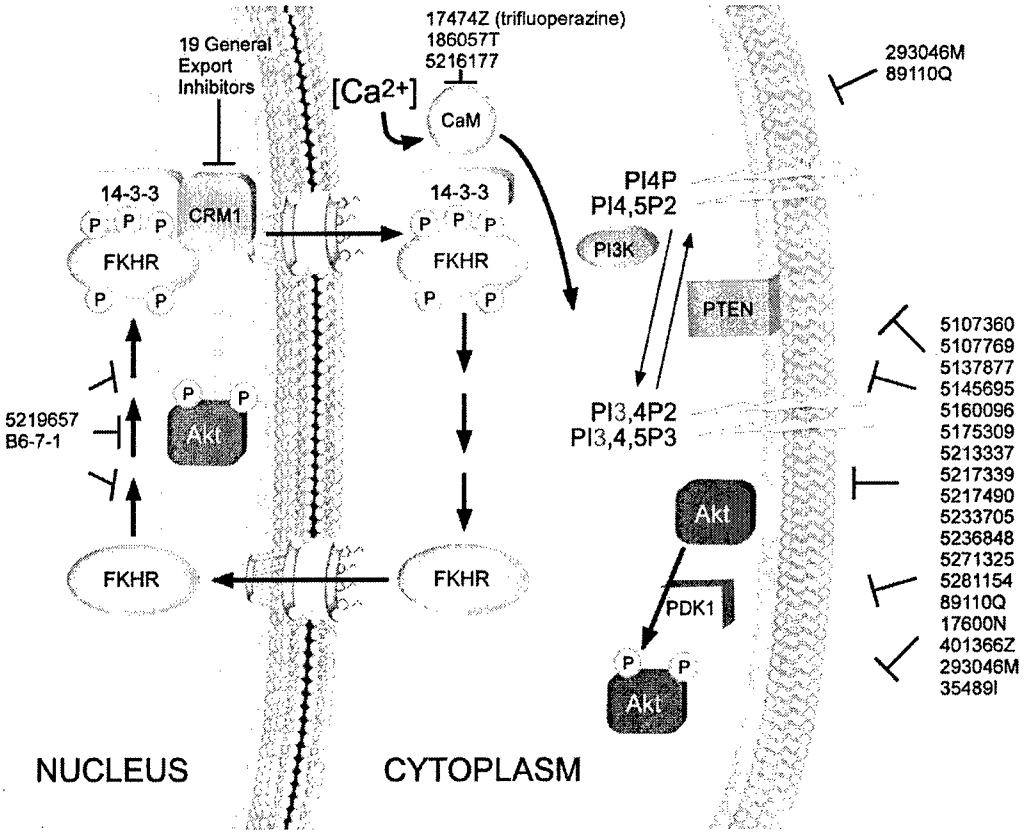


Figure 8. Target sites of compounds identified from the screen

Two inhibitors have targets located upstream of PI3K, 16 compounds target somewhere between PI3K and Akt inclusively, two inhibitors have targets downstream of Akt, 19 inhibitors target CRM1, and three inhibitors target CaM and implicate CaM as a novel regulator of FOXO1a activity and subcellular localization.

ments are currently underway to identify the targets of the remaining pathway-specific compounds.

Experimental procedures

Materials

LMB, wortmannin, and haloperidol were purchased from Sigma. L-SPD, W-13, calmidazolium, and ophiobolin A were purchased from Calbiochem. BAPTA-AM was purchased from Molecular Probes. L754870 was purchased from Tocris. Clozapine was purchased from Alexis Corp. Phenothiazine therapeutics thioridazine, fluphenazine, chlorpromazine, and prochlorperazine were purchased from ICN Biomedicals, Inc.

Lead compounds from the screen were ordered from ChemBridge Corp. or requested from the NCI. The pRev(1.4)-GFP+PKI NES plasmid was a gift from Beric Henderson and its construction described in Henderson and Eleftheriou (2000). The CRM1-Cys528Ser mutant plasmid, pXHCK1, was a gift from Minoru Yoshida and previously described in Akakura et al. (2001). The NLS-NES-GFP plasmid (pPS1372) was previously described in Taura et al. (1998).

Cell culture and yeast strains

786-O and U2OS-RevGFP cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Clone (Clonetech) and 100 μg/ml penicillin-streptomycin at 37°C, 5% CO_2. U2OS-RevGFP cells were established by transfecting U2OS cells with pRev(1.4)-GFP+PKI NES using FuGENE 6 reagent according to the manufacturer's protocol (Boehinger Mannheim). Stable clones were selected in complete media containing 400 μg/ml G418. Human mammary eptithelial cells, HMEC-Myr-p110α and HMEC-vector, were previously described in Zhao et al. (2003). Cells were grown in mammary epithelial basal medium (MEBM, BioWhittaker) supplemented with mammary epithelial growth medium (MEGM, BioWhittaker) consisting of hydrocortisone, EGF, insulin, and bovine pituitary extract.

Wild-type yeast PSY580 and CRM1 mutant yeast PSY1969 were transformed with the NLS-NES-GFP plasmid using a standard transformation protocol. Transformants were selected on ura$^-$ dropout plates.

Adenovirus construction

Ad-FKHR was generated with the pAD-Easy system (He et al., 1998). In brief, linearized shuttle plasmid containing the cDNA for FLAG-FOXO1a was cotransfected with pAdEasy-1 into BJ5183 cells. After isolation, recombinant adenoviral DNA was restriction digested with PacI and transfected into 293 cells. Infectious adenovirus was amplified in 293 cells. Purified virus was isolated by freeze-thaw extraction followed by CsCl gradient purification and titred by plaque lysis.

FOXO1a export assay in 786-O and HMEC cells

786-O cells were seeded onto 384-well, black, clear-bottom plates (Costar) at a density of ~2500–3000 cells/well in 50 μl complete media. After incubation at 37°C, 5% CO_2 for 2–3 hr, cells were infected with Ad-FKHR and incubated for ~24 hr. Small molecule compounds were serially diluted 1:2 starting from 80 μM in a separate 384-well plate using a 16-channel pipette (ThermoLabsystems) in DMEM. Media were aspirated from infected cells using a 24-channel wand before diluted small molecules were transferred onto cells. Cells were incubated for ~1 hr before 3.7% formaldehyde fixation. Fixed cells were then stained with M5 anti-FLAG antibody (Sigma) followed by washing with PBS three times and staining with Alexa Fluor 594 goat

anti-mouse antibody (Molecular Probes) and Hoechst 33258 (Sigma). Both antibodies, M5 and Alexa Fluor 594, were diluted 1:1000 in PBS/0.2%TX-100/5%FBS. For cell counts, at least 200 cells exhibiting nuclear, nuclear and cytoplasmic, or cytoplasmic staining were counted from three separate images. Percentages of N, N+C, and C cells were calculated and standard deviations determined.

HMEC's were treated similarly except they were seeded at a density of ~3500 cells/well and incubated overnight before infection with Ad-FKHR virus. Compounds were diluted in MEBM supplemented with MEGM.

High-throughput screen

Cell-based screening was performed at the Institute for Chemistry and Cell Biology (http://iccb.med.harvard.edu). 786-O cells were seeded onto black, clear-bottom, 384-well plates at ~2500–3000 cells/well in 50 μl complete media using a Multidrop liquid dispenser (Labsystems). Cells were then incubated for 3 or 17 hr before infection with Ad-FKHR for ~24 hr. Compounds from the ChemBridge DiverSetE (ChemBridge Corp.) and the NCI Structural Diversity Set (NCI) were kept at a stock concentration of 5 mg/ml (~10 mM) in DMSO. 100 nl of each compound, as well as those from the NCI marine extract plate, were transferred to cells by a solid 384-pin array device attached to a robotic arm. Cells were then incubated for ~1 hr at 37°C, 5% CO_2. Formaldehyde fixation and antibody staining were performed as described in the FOXO1a export assay.

Digital images of cells in each well were acquired using an automated fluorescence microscope equipped with a Plan Fluor 10X NA 0.3 objective (Nikon) and Metamorph software (Universal Imaging). FOXO1a subcellular localization was scanned visually for each imaged plate.

RevGFP export and CRM1 target assays

U2OS-RevGFP cells were seeded onto clear-bottom, black, 384-well plates at ~4500 cells/well in 50 μl complete media. Cells were allowed to attach and grow overnight before compound treatment. Compounds were serially diluted 1:2 starting from 40 μM in a separate 384-well plate in DMEM. Media were aspirated from cells before the diluted compounds were transferred onto cells. Cells were incubated with compound for ~1 hr before fixation with 3.7% formaldehyde and nuclei staining with Hoechst 33258. For the CRM1 target assay, U2OS-RevGFP cells were transfected with pXHCK1, the CRM1-Cys528Ser mutant, on a 10 cm plate using FuGENE 6 transfection reagent according to the manufacturer's directions. After ~17–24 hr of incubation, cells were detached and re-plated onto a black, clear-bottom, 384-well plate at ~4500 cells/well density. Cells were allowed to flatten and grow overnight before treatment with various compounds for ~1 hr. Fixing and staining procedures were similar to those used in the RevGFP export assay.

Yeast CRM1 assay

Wild-type PSY580 and CRM1 mutant PSY1969 yeast cells expressing the NLS-NES-GFP reporter (pPS1372) were grown to log phase in 10 ml YEPD media. Small molecule inhibitors were diluted in YEPD to a final concentration of 20 μM. 200 μl of cells were then aliquoted into individual wells of a 96-well, U-bottom plate. Cells were pelleted and resuspended in media containing diluted inhibitors and treated for ~1 hr at 30°C before fixation in 14% formaldehyde. Fixed cells were then washed twice and resuspended in 100 μl of Solution P (0.1 M potassium phosphate buffer [pH 6.5], 1.2 M sorbitol). 20 μl of cells was pipetted into each well of a black, polylysine-treated, 24-well slide. Cells were permeabilized with 0.5% NP-40 or Triton X-100 in Solution P before nuclei staining with 1 μg/ml DAPI in Aby Wash 2 (0.1 M Tris [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$). Coverslips were then mounted onto slides and cells visualized using an inverted fluorescence microscope with a 60× objective. Digital images were acquired using Metamorph software.

Phospho-Akt and phospho-SGK immunoblot

786-O cells were grown to confluency in 6-well tissue culture plates. Cells were then treated with compound diluted in 2 ml DMEM at decreasing concentrations, (e.g., 40, 20, and 10 μM) for ~1 hr at 37°C, 5% CO_2. Cells were then scraped in PBS, pelleted, and lysed in extract buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.5% deoxycholate, 1 mM PMSF, PLAC). Lysates were spun at 14,000 × g for 10 min and supernatants aliquoted, frozen on dry ice, and stored at −80°C. Protein concentrations were determined using Bradford reagent (Bio-Rad) and aliquots of lysates with equal amounts of protein were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in 5% powdered milk in PBST (PBS, 0.2% Tween-20), incubated overnight at 4°C in phospho-Ser473-Akt antibody (Cell Signaling) diluted 1:1000 in PBST containing 5% BSA. Phospho-Ser473-Akt levels were detected using anti-rabbit HRP diluted 1:5000 in 5% milk/PBST followed by Western Lightning chemiluminescence reagent (PerkinElmer). Blots were stripped in stripping buffer (100 mM glycine, HCl/pH 2.5), washed twice in PBST, and blocked in PBST containing 5% milk before re-probing with pan Akt antibody (Cell Signaling) diluted 1:1000 in PBST/5% BSA. Phospho-SGK (Upstate) was diluted 1:200 in PBST/5%BSA. For phospho-Thr308-Akt (Cell Signaling) immunoblots, membranes were blocked in 4% milk in TBST and secondary anti-rabbit HRP was diluted 1:3000 in 2% milk/TBST.

Cell viability assay

786-O cells were seeded onto opaque, white, 96-well plates (Costar) at ~2500 cells/well density and incubated for 24 hr. Compounds were serially diluted 1:2 from 40 μM to 1.125 μM on a different 96-well plate in serum-containing media. 100 μl of each dilution was transferred onto cells after removal of media. Cells were incubated with inhibitors for ~24 hr. Cell viability was assayed using CellTiter-Glo luminescent cell viablilty assay (Promega) following the manufacturer's protocol.

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The Biology and Clinical Relevance of the *PTEN* Tumor Suppressor Pathway

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ABSTRACT

Genetic alterations targeting the *PTEN* tumor suppressor gene are among the most frequently noted somatic mutations in human cancers. Such lesions have been noted in cancers of the prostate and endometrium and in glioblastoma multiforme, among many others. Moreover, germline mutation of PTEN leads to the development of the related hereditary cancer predisposition syndromes, Cowden disease, and Bannayan-Zonana syndrome, wherein breast and thyroid cancer incidence is elevated. The protein product, PTEN, is a lipid phosphatase, the enzymatic activity of which primarily serves to remove phosphate groups from key intracellular phosphoinositide signaling molecules. This activity normally serves to restrict growth and survival signals by limiting activity of the phosphoinositide-3 kinase (PI3K) pathway. Multiple lines of evidence support the notion that this function is critical to the ability of PTEN to maintain cell homeostasis. Indeed, the absence of functional PTEN in cancer cells leads to constitutive activation of downstream components of the PI3K pathway including the Akt and mTOR kinases. In model organisms, inactivation of these kinases can reverse the effects of PTEN loss. These data raise the possibility that drugs targeting these kinases, or PI3K itself, might have significant therapeutic activity in PTEN-null cancers. Akt kinase inhibitors are still in development; however, as a first test of this hypothesis, phase I and phase II trials of inhibitors of mTOR, namely, rapamycin and rapamycin analogs are underway.

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INTRODUCTION

Cancer-causing genetic alterations fall broadly into two functional classes: those that activate cellular genes, known as oncogenes, and those that inactivate cellular genes, known as tumor-suppressor genes. Growing evidence suggests that inactivation of the tumor suppressor gene *PTEN* may rival mutations of *p53* in frequency and in the relevance to a substantial fraction of adult epithelial tumors. Emerging therapeutics that may prove to be of particular utility in treating tumors lacking PTEN function are under clinical development—thus the impetus for providing a framework within which such inhibitors can be understood.

In 1997, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), also known as *MMAC1* and *TEP1*, was cloned and mapped to cytoband 10q23, a region undergoing frequent somatic deletion in tumors.[1-3] In two instances, groups specifically searching either in glioma or breast cancer for a 10q23 tumor suppressor happened on the same gene, hence the alternative names.[1,3] *PTEN* is the accepted gene symbol and will be used henceforth.

GERMLINE MUTATION OF PTEN AND COWDEN SYNDROME

Germline mutations of tumor suppressor genes are often associated with hereditary cancer predisposition syndromes. Included among the many are the hereditary breast and ovarian cancer syndromes associated with germline mutation of *BRCA1* and *BRCA2* and hereditary nonpolyposis coli associated with mutations in *MLH1* and *MLH2*. Such syndromes, though inherited in an autosomal dominant pattern, result from a recessive mutation (loss-of-

function) of the tumor suppressor gene in question.

Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS) are related hamartoma syndromes. Affected members within CS kindred develop hamartomas of the hair follicle (trichilemmomas), the mucocutaneous membranes, breast, thyroid, and intestinal tissues—detailed criteria are reviewed by Eng et al[4]—whereas features of BRRS include macrocephaly, lipomatosis, hemangiomatosis, and speckled penis.[5] These kindred are at high risk for developing cancers of the breast and thyroid and more recently endometrial and genitourinary tract tumors were included as minor criteria in CS.

In 1996, a cooperative group reported linkage analyses mapping the genetic locus for Cowden syndrome to the 10q23 region.[6] The cloning of *PTEN* was quickly followed by the discovery of germline *PTEN* mutations in 80% of families with CS and 60% of subjects affected by BRRS.[7-10] Thus, as with other tumor suppressor genes, inheritance of a germline *PTEN* mutation results in the initiation of a cancer susceptibility syndrome.

SOMATIC MUTATION OF PTEN

Germline mutations constitute a minor fraction of the alterations in tumor suppressor genes that contribute to the pathogenesis of human tumors, and instead are often useful heralds for the discovery of somatic mutations in sporadic nonfamilial tumors. Somatic alteration and biallelic mutation of *PTEN* is indeed a common event in high-grade glioblastoma, melanoma and in cancers of the prostate and endometrium.

Biallelic inactivation of *PTEN* occurs in 30% to 40% of glioblastoma multiforme and to a lesser extent in anaplastic astrocytoma, yet is rarely seen in lower-grade glioma and glioneuronal tumors.[11-16] Recently, Smith et al reported *PTEN* mutations in 11 of 62 anaplastic astrocytomas (18%) and in 37 of 110 GBMs (34%). Moreover, tumors harboring any *PTEN* alteration were associated with a significantly shorter median survival (10.4 months *v* 14.7 months; $P < .001$). Thus in glial tumors the frequency of *PTEN* mutation increases with tumor grade and is associated with a poor outcome.[16]

Knock-out mice rendered heterozygous for *pten* develop a number of neoplasia including those of the endometrium,[17,18] and up to 50% of unselected human endometrial cancers (EC) harbor *PTEN* mutations. This rate approaches 80% to 90% in the endometrioid sub-type making *PTEN* the most commonly mutated gene in EC.[19-23] In contrast to glioma, mutation of *PTEN* is also seen in EC precursor lesions including endometrial hyperplasia and atypical hyperplasia suggesting that *PTEN* loss is an early event in this disease.[22,23] Intriguingly and again in contrast to glioma, in EC loss of *PTEN* is associated with improved survival.[24]

As mentioned above, the 10q23 region is a frequent target for heterozygous deletion in primary and more frequently in metastatic prostate tumors where loss-of-heterozygosity (LOH) is found in 20% to 60% of such tumors.[25] In keeping with these data, point mutations or deletions of the *PTEN* gene have been reported in cell lines, prostate cancer xenografts and in primary and metastatic deposits.[26-32] The rate of second mutational events varies widely and is generally less frequent than the incidence of LOH; however, second *PTEN* mutations are found in the tumor deposits of as many as 50% of patients with metastatic disease.[33] In addition, loss of the PTEN protein occurs in 20% of primary prostate tumors and this loss is highly correlated with advanced tumor grade and stage (Gleason score > 7).[34] These data suggest that there is progressive loss of PTEN or accumulation of mutations in the *PTEN* gene in association with advancing disease.

Loss or mutation of *PTEN* is high in malignant melanoma cell lines,[35] and as is the case with prostate cancer there is discordance between the rate of LOH at 10q23 found in primary melanoma specimens and the presence of secondary mutations. Although *PTEN* mutations do occur in metastatic melanoma samples the frequency has ranged from 7% to 19%.[36-40] Thus, when *PTEN* is lost there is again a correlation with more advanced disease.

PTEN mutations have also been found, though to a lesser extent, in cancers of the bladder, lung, ovary, colon, and lymphatic system.[31,41-44]

IS THERE EPIGENETIC INACTIVATION OF PTEN?

In a number of cancers the rate of hemizygous inactivation events (LOH) in the 10q23 region significantly exceeds the rate of mutation of the remaining *PTEN* allele. For example, though germline *PTEN* mutations in CS predispose to thyroid cancer and breast cancers, only infrequent *PTEN* mutations (6% to 7%) have been detected in the corresponding sporadic thyroid or breast carcinomas.[45-49] Similarly, mutations of *PTEN* in ovarian cancer are relatively rare though endometrioid sub-types of ovarian cancer may undergo *PTEN* loss at a greater frequency.[50-52] In CS-related tumor types hemizygous inactivation of *PTEN* occurs with impressive frequency, yet loss of the second allele is rare.

The tumor suppressor paradigm characteristically calls for loss of both functional copies of the gene and indeed many, but not all tumor suppressor genes must undergo biallelic inactivation to sustain a true loss-of-function effect. Thus, discordance between the rate of LOH and the rate of mutation of the second allele has led some to suggest that a second tumor suppressor gene is harbored in the 10q23 region. However, this difference could also result from the technical inability to detect second mutational events (low sensitivity); a gene dosage effect where loss of

one allele of *PTEN* may have a partial tumor promoting effect; cooperation between loss of one allele of *PTEN* and a genetic event in a second gene or finally from epigenetic alterations in the *PTEN* gene, mRNA, or protein leading to a true loss-of-function.

Epigenetic inactivation of the *PTEN* promoter was first described in prostate cancer xenografts[32] where loss of PTEN protein was accompanied by promoter methylation. Although promoter methylation in primary tumor specimens has not been demonstrated, loss of the protein and loss of the PTEN mRNA does occur. For example, immunohistochemistry studies in tumors where LOH is common, but second mutations are rare, including thyroid, breast, pancreatic and ovarian cancers have demonstrated loss of PTEN protein in 30% to 50% of samples.[47,53-56] In breast cancer this loss strongly correlates with lymph node metastasis and with estrogen receptor–negative tumors.[54] Moreover, in thyroid and ovarian cancer such loss is accompanied by concordant activation of the PTEN regulated signaling pathway and re-expression of PTEN in thyroid tumor cell lines markedly inhibits cell growth.[47] Together these data suggest that loss of PTEN as detected by Immunohistochemistry is functionally relevant.[47,55]

Although promoter methylation is the most common epigenetic mechanism for loss of gene expression, alternative mechanisms could contribute to the downregulation of the *PTEN* mRNA and/or protein. For instance, tumors might have acquired mutations in noncoding regions of *PTEN*, as yet unanalyzed, that are required for the expression of the transcript. In CS, for example, nonsense mutations leading to the degradation of the mRNA have been reported.[7,8,10] *PTEN* mRNA can also be regulated through TGF-β and through p53.[2,57] The TGFβ pathway is deregulated in a number of cancers, and in pancreatic cancer overexpression of TGFβ1 appears to be highly correlated with reduced PTEN levels.[56]

The PTEN protein is also regulated by phosphorylation.[58] In particular, the serine/threonine kinase CK2, upregulated in many cancers, phosphorylates the PTEN C-terminal tail and reduces PTEN activity[59-61] raising the possibility that phosphorylation of PTEN by CK2 might be a mechanism contributing to the downregulation of PTEN in certain tumors retaining a wild-type *PTEN* allele.

PTEN AND THE PI3K PATHWAY

PTEN: A Lipid Phosphatase

The protein encoded by the *PTEN* gene is a phosphatase—an enzyme that facilitates the removal of phosphate groups from macromolecules (dephosphorylation). Though PTEN can dephosphorylate proteins,[62] its primary biochemical and physiological targets are highly specialized plasma membrane lipids[63] (Fig 1). These lipids, phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4-bisphosphate are produced during cellular signaling events by the action of the lipid kinase phosphoinositide 3-kinase (PI3K) (for review see reference[64]). Thus, an elegant on-off switch has been evolved where the switch moves to "on" position when PI3K deposits a phosphate group on the D3 position of the inositol ring and is turned "off" when PTEN removes the phosphate group from the same position.

A critical role for this switch in both the normal response to growth signals and in the abnormal response to transforming signals was evident in the mid 1980s when PIP3 was first discovered as an evanescent molecule, the abundance of which was significantly upregulated on growth factor stimulation. At the same time, elevated PI3K activity was linked both to transformation by oncogenes, such as polyoma middle T antigen, and to mitogenic stimulation through the platelet derived growth factor receptor.[65-67]

The discovery of PTEN's lipid phosphatase activity, and its ability to act as an "off" switch for PI3K signaling, suggested that PTEN functioned as a tumor suppressor by directly antagonizing the activity of the PI3K signaling pathway.[63] Indeed, the past several years have witnessed the production of an impressive body of experimental data supporting this model.

The Phosphoinositide 3-kinase/Akt Pathway

Signaling through the PI3K pathway begins with the receipt of cell growth and survival signals sensed and relayed to the internal cellular environment by receptor tyrosine kinases (RTKs) spanning the plasma membrane. On ligand activation, RTKs engage and activate the PI3K holoenzyme resulting in the recruitment of PI3K to the membrane and the generation of PIP3 (Fig 2). The epidermal growth factor receptor the target of gefitinib (Iressa; AstraZeneca, Wilmington, DE); the Her2/*neu* receptor targeted by Herceptin; c-kit a target of imatinib (Gleevec; Novartis, Summit, NJ) and the insulin-like growth factor receptor 1 are among the many RTKs that can engage the PI3K signaling pathway in this manner.

Once generated, the phospholipid PIP3 serves as a nidus for recruiting certain kinases to the plasma membrane including the Protein kinase B/Akt family of kinases and phosphoinositide-dependent kinase 1 (PDK1).[68,69] On membrane localization Akt is activated, in part through phosphorylation by PDK1, and is then capable of phosphorylating a number of downstream targets (Fig 2). These Akt targets or substrates play key roles in regulating critical cellular functions including proliferation, apoptosis, glucose homeostasis, cell size, nutrient response and DNA damage.

As predicted by this model, genetic inactivation of *PTEN* in human cancer cell lines, in mouse knock-out models and in lower organisms including *C elegans* and *D melanogaster*, leads to constitutive activation of this pathway[70]. Moreover, in each of these systems, specific cellular

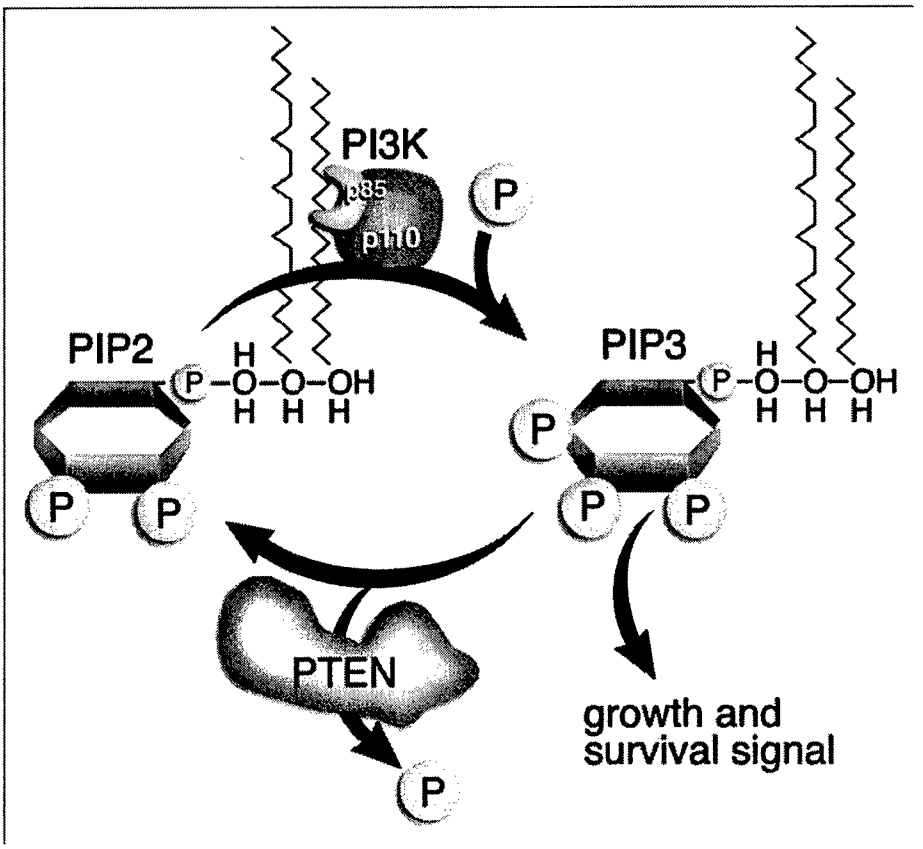

Fig 1. PTEN (blue), a lipid phosphatase. Phosphoinositide 3-kinase (PI3K; green), is a lipid kinase catalyzing transfer of a phosphate group (yellow) to phosphatidylinositol-4,5 bisphosphate (PIP2), generating phosphatidylinositol-3,4,5 trisphosphate (PIP3), which transmits growth and survival signals. PTEN removes D3 phosphate from PIP3, inactivating the signaling cascade and regenerating PIP2.

or organismal phenotypes resulting from PTEN loss can be accounted for and, in many cases, reversed by alterations in PI3K or Akt activity or by alterations in further downstream members of the pathway. Simply put, loss of PTEN results in alterations in cell homeostasis that depends on the activity of the PI3K pathway.

The Role of the PI3K/Akt Pathway in Oncogenesis

As is often the case for cellular tumor suppressor pathways, the PI3K/Akt pathway is targeted and dysregulated by a number of retroviral or DNA tumor viral oncogene products. As mentioned above the role for this pathway in transformation was first brought to the fore by the finding that middle T antigen, a transforming oncoprotein produced by polyoma virus, binds to and activates PI3K independently of growth factor signaling.[65] In addition, retroviral oncogenes that render the catalytic subunit of PI3K or Akt constitutively active were discovered in avian and murine tumors.[71-73]

Similarly, in human tumors loss of PTEN function appears to be only one of a number of different genetic alterations used by tumors to constitutively activate the PI3K pathway, presumably indicating a selective growth or survival advantage accrued to tumor cells harboring such lesions. The gene *PIK3CA*, encoding the catalytic subunit of PI3K (p110), is located in a common amplicon at 3q26. Amplifications of this region have been reported in cancers of the ovary, cervix, head and neck and at least in certain cases, when examined in detail, such amplifications have been associated with increased PI3K activity.[74-78] p85α, one of the regulatory subunit of PI3K, undergoes mutation that likewise is predicted to render the holoenzyme constitutively active.[79,80] Similarly, amplifications of *AKT* kinase genes, notably *AKT2*, have been reported, albeit at low frequency in ovarian, pancreatic, breast, and gastric cancers.[73,81-84]

Finally, as discussed in greater detail below, recent data suggests that the tumor suppressor genes tuberous sclerosis 1 (*TSC1*) and tuberous sclerosis 2 (*TSC2*) are also key regulators of a pathway known as the mTOR pathway (mammalian Target of Rapamycin) which is, at least in part, a downstream component of the PI3K pathway (Fig 3). Thus, hereditary and possibly somatic loss-of-function mutations in these two genes are yet another means by which downstream PI3K pathway events can be activated.

IGF-1 Signaling Pathway and PTEN

Among the many upstream tyrosine kinase receptors that can activate PI3K the insulin receptor family is perhaps the most tightly linked. Among the data forging this link are studies of aging and nutrient response in *C elegans* where

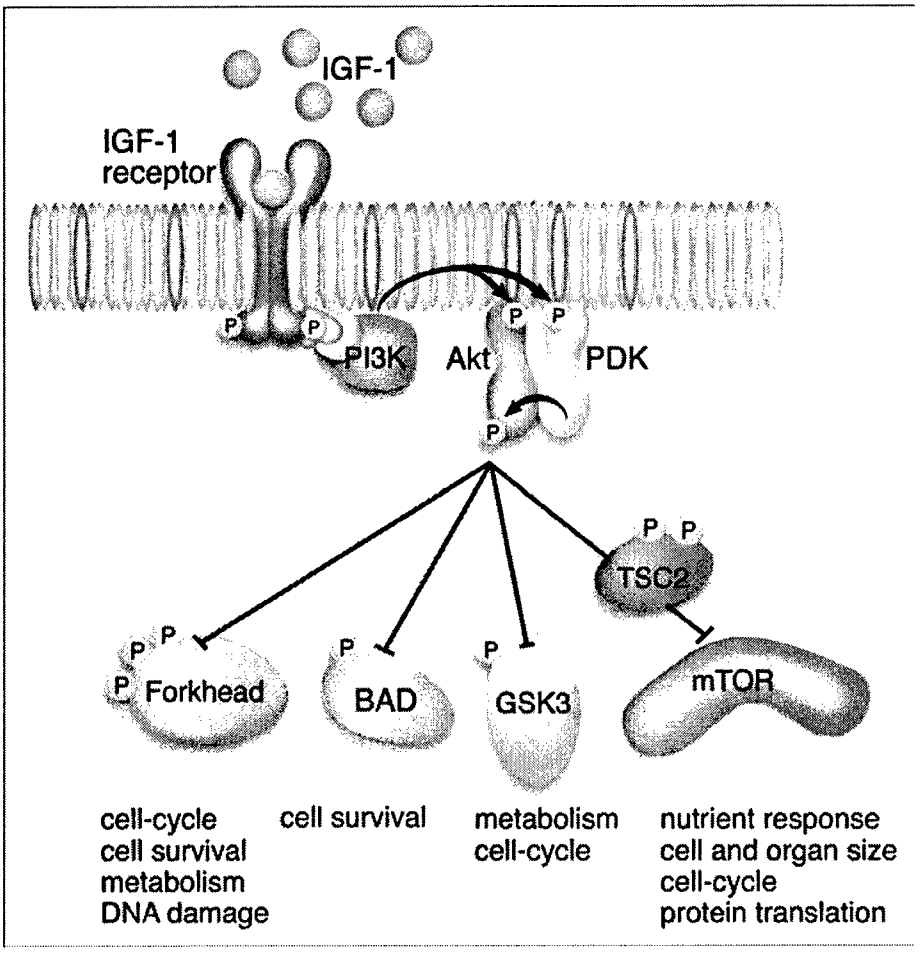

Fig 2. Binding of growth factor ligands activates kinase receptors leading to recruitment of PI3K to receptor complex. Activated PI3K phosphorylates PIP2 (blue), which in turn activates PDK1 and Akt, leading to phosphorylation and inhibition of downstream substrates.

the pi3k pathway is highly conserved and where insulin-like growth factor (IGF) signaling is genetically linked to *pten*, *Akt*, and *pi3k*. In mammalian cells, IGF-1 does indeed signal through this pathway. Data from prospective studies of serum and nutrient risk factors for cancer have linked IGF-1 serum levels to risk for a number of cancers including those of the breast, colon, and prostate.[85] Given that germline alterations in *PTEN* lead to elevated cancer rates in affected individuals an intriguing possibility is that cancer risk and IGF-1 levels are tied to chronic alterations in the levels of PI3K activity in target organs. If so, therapeutic strategies applicable to the treatment of tumors dependent on PI3K signaling may also find a role in prevention strategies.

PTEN/PI3K/AKT PATHWAY REGULATES FUNDAMENTAL CELLULAR PROCESSES LINKED TO TUMORIGENESIS

The signaling outputs of the PI3K pathway, through Akt and other effectors, lead to alterations in multiple cellular processes including cell-cycle regulation, cell-survival, cell adhesion and motility, angiogenesis, glucose homeostasis, and cell size and organ size control. Each is deserving of attention; however, recent developments elucidating connections between PTEN/PI3K/Akt and mTOR signaling make this area worthy of greater detail.

Regulation of the Cell Cycle Progression

Both loss of function studies in mice and reconstitution experiments in mammalian cells have shown that PTEN is a key regulator of progression through the mammalian cell cycle. Though typically thought of as a survival factor, multiple lines of evidence suggests that Akt may be the major downstream target for PTEN mediated G1 arrest. Among the Akt substrates thought to play an important role in cell cycle control are the Forkhead transcription factors FKHR, AFX and FKHRL1, GSK3 and substrates, such as TSC2, that play a role in regulating mTOR signaling.[86-89]

Regulation of Cell Survival

Loss of PTEN function in vitro or in vivo, or activation of Akt results in alterations in cell survival. For example, *pten*−/− murine fibroblast cells are impaired in their response to apoptotic stimuli[90] and *pten*± mice develop a

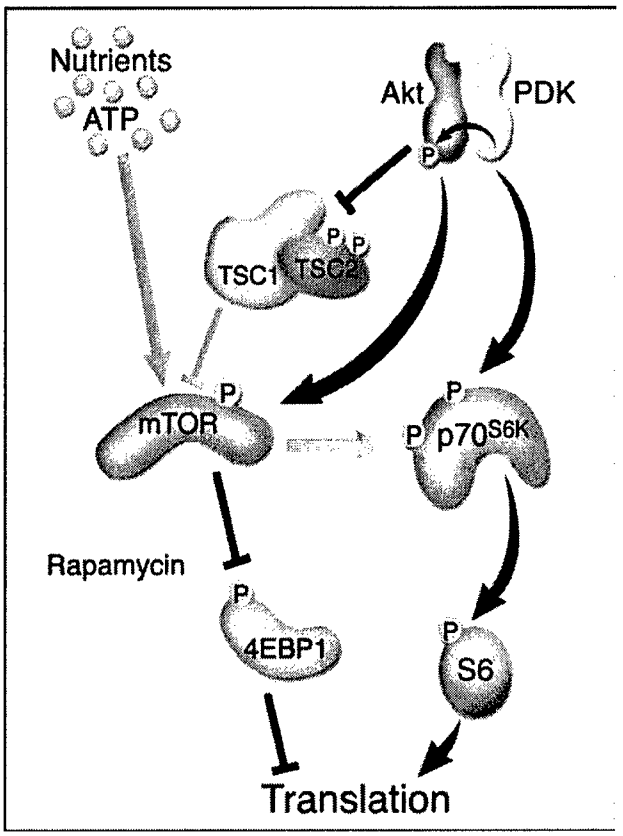

Fig 3. mTOR pathway, which can receive upstream inputs from both the PI3K pathway and unknown sensors of nutrients, glucose, or energy. The PI3K signal can be transmitted through phosphorylation of TSC2 by Akt, leading to inhibition of TSC2 and then activation of mTOR; through Akt phosphorylation and activation of mTOR; or through PDK1 phosphorylation of p70S6K. Arrows and T lines indicate activating and inactivating connections, respectively. Connections where mechanism is known (black) and where mechanism is unknown (gray).

lymph-node hyperplasia syndrome resulting from defects in FAS-mediated apoptosis.[17,91] Among Akt substrates linked to the regulation of apoptosis are the forkhead transcription factors that can activate the transcription of the pro-apoptotic genes *FAS* and *Bim*.[92,93] Akt also phosphorylates and inactivates the pro-apoptotic Bcl-2 family member Bad.[94]

Regulation of Cell Spreading and Motility

PI3K transmits signals not only to Akt, but also to a number of other downstream effectors including the Rho family of GTPases (Rho, RAC1, and cdc42) that are key mediators of membrane ruffling, cell motility, and cell spreading. As a result, loss of one gene copy of *PTEN* increases the motility and invasiveness of murine embryonic fibroblasts or mammalian cancer cells, while reexpression of PTEN abrogates this effect.[95-98] These data raise the possibility that the notable increase in the *PTEN* mutation rate in metastatic tumors might result from a selective metastatic advantage acquired through the loss of PTEN regulation of motility and invasion.

Regulation of Angiogenesis

Emerging data suggests that PTEN suppresses the hypoxia-mediated stabilization of hypoxia inducible transcription factor 1 (HIF1). HIF1, when stabilized, upregulates the expression of vascular endothelial growth factor a potent stimulator of new blood vessel formation.[99] Thus, loss of PTEN or activation of PI3K/Akt may also endow tumors with angiogenic properties.

Regulation of mTOR (mammalian target of rapamycin) Signaling: A Nutrient Response Pathway

The mTOR pathway functions as part of a nutrient sensing mechanism regulating the cellular response to starvation or growth conditions such as amino acid deprivation. Emerging data have demonstrated a significant role for PTEN in controlling cell size, organ size, and proliferation through regulation of the mTOR pathway. Notably in *D melanogaster* loss-of-function mutants in the *Drosophila PTEN* homolog (*dpten*) result in increased cell and organ size, while overexpression of dpten yields the opposite phenotype.[100-102] Similarly, mice specifically lacking *pten* in neuronal cells develop a syndrome of CNS enlargement, neuron enlargement, seizures, ataxia, and premature death[103,104] markedly similar in these features to Lhermitte-Duclos disease, a human neurologic disorder that is a component of Cowden syndrome and is thus caused by germline PTEN mutation.[105]

Recently, genetic studies in *D melanogaster* have also placed the gene products (hamartin and tuberin) of the tuberous sclerosis genes (*tsc1* and *tsc2*) in the dTOR pathway. Here, as with *dpten*, loss-of-function mutations in the drosophilia homologues *dtsc1* and *dtsc2* lead to increased cell size, proliferation, and deregulated organ size.[106] Tuberous sclerosis, like CS and BRRS, is an autosomal dominant disorder characterized by hamartoma formation in a variety of tissues including the brain, skin (not the hair follicle), and kidney leading to a set of common clinical symptoms including seizures, mental retardation, autism, kidney failure, facial angiofibromas, and cardiac rhabdomyomas.[107,108] Mutation in either the *TSC1* or *TSC2* tumor suppressor gene is responsible for both the familial and sporadic forms of this disease. Thus, two hamartoma syndromes, with distinct phenotypes resulting from mutation in different genes (*PTEN* and *TSC* genes), are both a result, at least in part, from deregulated activation of the mTOR signaling pathway.

Although the biologic bases of these pathologic alterations are not well understood, the molecular connections are becoming more evident (Fig 3). Epistasis studies in *D melanogaster* have demonstrated that *dpten*, *dtsc*1, and *dtsc2* act upstream of *ds6k* (a gene encoding a kinase known as p70S6K in mammalian cells) and while *dpten* appears to be upstream of *dAkt*, *dtsc1*, and *dtsc2* are downstream of

dAkt.[109] Moreover, in various organisms, recent data have shown that the *TSC2* gene product is a direct substrate of Akt that is inhibited by such phosphorylation events and thus can modulate PI3K-dependent activation of p70S6K.[110,111] Although the precise action of the *TSC* genes are not known it is clear that they, like PTEN, are negative regulators of signaling through mTOR and in turn p70S6K.

The mTOR pathway has two key downstream components germane to the study of human tumors, the ribosomal protein S6, a direct substrate of p70S6K and 4EBP-1, an inhibitor of protein translation. Due to increase in mTOR and in p70S6K activity, both are aberrantly phosphorylated in PTEN-null cells. Thus, while mTOR and p70S6K may serve as targets for antineoplastic agents, S6 and 4EBP-1 may serve as markers of pathway activity in human tumors. Finally, emerging and older data have suggested that specific members of the mTOR pathway, including p70S6K may themselves be targeted for oncogenic activation.[112]

THERAPEUTIC APPROACHES TARGETING THE PI3K PATHWAY

It is clear that deregulated activation of the PI3K/Akt pathway, achieved through the numerous genetic and epigenetic alterations, contributes substantially to the pathogenesis of a growing list of human cancers. Although direct genetic alteration of kinases (eg, *BCR-ABL*) in human cancer is one mechanism by which cells may be rendered "kinase" dependent, kinase activation and dependency might also occur through genetic inactivation of kinase regulators such as PTEN. Indeed, emerging data suggests that tumors harboring such alterations likely remain dependent on a persistent PI3K signal for continued proliferation, survival, or migration. Such dependency may point to a substantial therapeutic window for small molecule inhibitors developed to interdict PI3K signal transmission. Thus, there is guarded optimism that successful therapeutics direct against certain kinase elements of the PI3K pathway will be developed. Optimism that is, at least in part, based on the already proven ability to develop relatively specific, small molecule kinase inhibitors, such as imatinib and gefitinib.

Inhibition of PI3K

In the laboratory, investigators commonly use two PI3K inhibitors, wortmannin and LY294002 as experimental reagents. Both have demonstrated marked antitumor cell activity, particularly in PTEN-null cells or in cells overexpressing PI3K.[113,114] These inhibitors tend to be relatively broad in their activity and can render inactive a host of kinases related to PI3K including ATM and ATR. Moreover, recent evidence suggests that even among the PI3K family members, one might gain therapeutic advantage by directing molecules more selectively against the p110α protein. To date, however, no additionally selective inhibitors have been described and we are unaware of any published clinical data for the use of wortmannin or LY294002 in humans.

Inhibition of Akt

In mouse embryonic stem cells inactivation of *Akt1* significantly attenuates tumor formation resulting from loss of *pten*[115] and, in mammalian cells, restoration of the activity of the Akt substrate FKHR reverses the transformed phenotype of PTEN-null cells[116] together suggesting that inactivation of Akt may have therapeutic benefit. A number of industry programs are actively pursing the development of Akt inhibitor though none are as yet in the clinic.

Receptor Tyrosine Kinase Inhibitor

It is likely that activation of RTKs, as upstream activators of the pathway, would cooperate with loss of PTEN to provide a robust constitutive PI3K signal. If so, then PTEN-null tumors may yet depend on such activity for their growth thus leaving open the door for therapeutic inhibition of RTKs using agents such as gefitinib or novel therapeutics against the IGF-1 tyrosine kinase receptor (as examples) in these tumors. Alternatively, loss of PTEN may render tumor cells independent of such upstream activation events and therefore immune to this therapeutic strategy. Indeed, certain PTEN-null cells initially resistant to epidermal growth factor receptor inhibition regain responsiveness to gefitinib to combined on restoration of PTEN function.[117]

Inhibitors of mTOR: Rapamycin and Its Analogs

As mentioned previously, deregulated signaling through the mTOR pathway is a prominent consequence of PTEN inactivation. mTOR was originally identified as the target of rapamycin, a natural antibiotic derived from the organism *Streptomyces hygroscopicus* found on Easter Island (also known as Rapa Nui).[118] Through this action rapamycin has been known to block T-cell activation, arrest cell proliferation and thus is being developed as an immunosuppressant for use following kidney transplantation. At least two esterified and thus orally available rapamycin derivatives are in development CCI-779 (Wyeth Research, Madison, NJ) and RAD001 (Novartis).

The link between PTEN and mTOR suggested that PTEN-null cells might require mTOR activation for maintenance of aspects of the transformed phenotype including proliferation. Indeed, PTEN-null tumor cell lines, xenografts and tumors in mice appear to have selective sensitivity to rapamycin and CCI-779[119,120] raising the possibility that such compounds might have a therapeutic role in patients whose tumors lack PTEN.

As described earlier, loss of PTEN function is associated with phosphorylation and activation of Akt, p70S6K, mTOR, and 4E-BP. Thus, analysis of activation of downstream targets of PTEN may identify a broader spectrum of patients responsive to the effect of CCI-779 than would

mutation analysis alone. In fact cells transformed by v-Akt or v-PI3K, but not by a host of other oncogenes, are also quite sensitive to rapamycin.[121]

Clinical data for RAD001 are scant as phase I testing is still in progress. CCI-779 has been administered to patients both orally and intravenously in both phase I and phase II trials. Dose-limiting toxicity (stomatitis, rash, and increased AST) was reached at 100 mg PO daily for 5 days every 2 weeks[122] and a maximum tolerated dose for intravenous (IV) administration weekly was not reached in one study at a dose level of 220 mg/m^2 [123] though dose-limiting hypocalcemia was seen at high doses in another.[124] In the initial phase I trials, several patients with renal cell cancer experienced tumor regression,[123] a response has been noted in metastatic breast cancer and disease stabilization non–small-cell lung cancer, sarcoma, mesothelioma, and renal cell cancer.[124] Adverse effects include rash, mucositis, asthenia, alterations in liver function tests, leukopenia and thrombocytopenias. Phase II clinical trials are underway in glioma (NABTC 0101), prostate, metastatic breast cancer, renal cell carcinoma, lymphoma, melanoma, and small cell lung cancer (ECOG 1500). In 110 patients with renal cell carcinoma the preliminary results of CCI-779 treatment were recently reported. CCI-779 was well tolerated with frequent mild side effects of rash (72%) and mucositis (65%) while the most frequent serious side effects were hyperglycemia and anemia. Here, 5% of patients had partial responses, while a larger fraction had stable disease.[125] Similar side effects have been reported in a phase II trial in metastatic breast cancer where in the first 85 evaluable patients, stable disease or partial response for $\geq$ 3 months was observed in seven patients treated at 75 mg and 10 patients treated at 250 mg IV weekly.[126]

The doses tested in the studies of CCI-779 have generally ranged on the high side 75 to 250 mg IV weekly or 75 mg PO daily (5 days out of 14 days). An essential question is whether such doses exceed the maximally effective bioactive dose, in other words, the minimal dose required to maximally inhibit mTOR. If so, then excessive dosing might lead to side effects unrelated to inhibition of the mTOR pathway (in other words unrelated to the putative therapeutic mechanism). In addition, these studies have yet to report the activation status of the PI3K or mTOR pathways or the status of PTEN in these patients, thus the hypothesis that such tumors are, in humans, hypersensitive to such inhibition remains untested. If the data from animal and cell–based models is indicative of the response of PTEN-null tumors in humans then a reasonable prediction is that lower doses of mTOR inhibitors combined with rigorous patient selection might lead to greater efficacy with lower toxicity.

Substantial preclinical data have established the PTEN/PI3K/Akt pathway as a major oncogenic pathway linked to the development of some of the most common human cancers. The future holds great promise for the rapid development of selective novel anticancer agents specifically targeting components of this pathway. Understanding the parameters for patient selection and developing pharmacodynamic markers that allow for the optimizing of drug dose and schedule will likely aid in swinging the balance from toxicity to therapeutic effect.

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Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Acted as a consultant within the last 2 years: William R. Sellers, Novartis, Pharmacia. Received more than $2,000 a year from a company for either of the past two years: William R. Sellers, Novartis, Pharmacia.

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